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A Method of Estimating the Circulating Blood Volume by Sodium Para-aminohippurate

2nd Report

By

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The subject method, published in the previous report[1] by the author (former name Takashi Kishine) and his collaborators, is based on their empirical knowledge that, in normal persons, the ratio of the PAH quantity in the circulating plasma to the total PAH quantity retained in the body 30 min. after the injection of 2,000 mg. of the chemical gives an approximately fixed value of 0.153. Consequently, the method is applicable only to normal subjects, but not to pathological cases. In subsequent studies, I have succeeded in contriving a method presumably applicable to pathological cases as well, which I will report here.

Theoretical Consideration

Upon measuring the PAH concentration in the circulating plasma 5, 10, 15 and 20 min. (point of time t_1, t_2, t_3 and t_4) after the injection of 2,000 mg. of PAH, I found that the logarithms of the concentration values of PAH in the circulating plasma decreased in an approximately linear relation against the time t, as we have seen in the previous report[1] in the PAH equilibrium.

Now in Fig. 1, take the time range of t_1–t_4 and let x stand for the PAH quantity lost into urine, y for its quantity absorbed by tissues from the circulating plasma and z for the decrease in the PAH quantity present in the circulating plasma, in the length of time t, then

$$z=x+y$$

so

$$dz=dx+dy \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

Because the logarithms of the PAH concentration c in the circulating plasma fall in a straight line when plotted against the time t, this straight line can be illustrated in the following equation:

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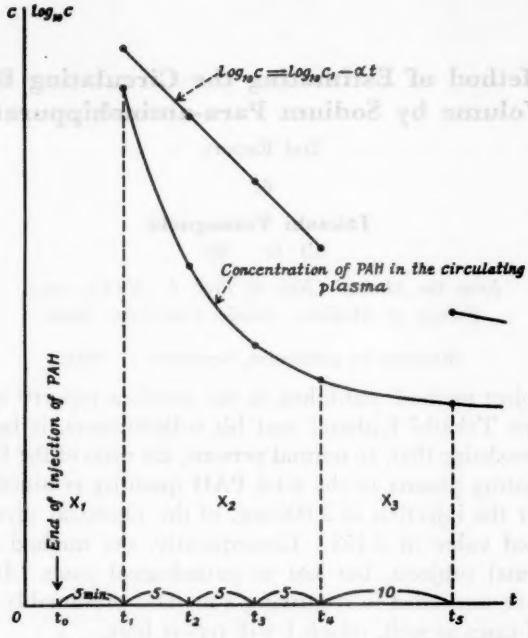

Fig. 1. Relation of concentration of PAH in the circulating plasma and of its logarithm to the time t.

$$\log_{10} c = -\alpha t + C \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

wherein C is a constant and $-\alpha$ is the direction coefficient.

Take t_1 as the starting point of time, and let c_1 be the PAH concentration in the circulating plasma at t_1, and then from (2)

$$C = \log_{10} c_1$$

Therefore, (2) may be rewritten as:

$$\log_{10} c = \log_{10} c_1 - \alpha t \quad \ldots\ldots\ldots\ldots\ldots (3)$$

When the total PAH quantity in the circulating plasma is a at the point of time t_1; the following may be readily deduced from (3):

$$\log_{10} (a-z) = \log_{10} a - \alpha t$$

Differentiating in respect to t, we get

$$\frac{dz}{dt} = (a-z)\alpha \log 10 \quad \ldots\ldots\ldots\ldots\ldots (4)$$

Let V_p represent the circulating plasma volume and v the renal plasma flow, and as reflected in our previous report[1)]

$$\frac{dx}{dt}=\frac{0.88v\,(a-z)}{V_p}$$

Therefore, from (1) and (4), it follows that $\frac{dy}{dt}$ must be also a linear function of (a−z). That is, the transition of PAH from the circulating plasma into the tissues is in direct ratio to its concentration in the circulating plasma.

From (3), it is seen that $c=10^{-at}\,c_1$, so the PAH quantity (X_2) lost into the urine during the time range t_1-t_4 is represented as follows:

$$X_2=0.88v\int_0^{15} cdt=0.88vc_1\int_0^{15} 10^{-at}dt$$

$$=\frac{0.38vc_1}{a}\,(1-10^{-15a}) \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

and X_3 for the time range t_4-t_5 (t_5 stands for the point of time just 30 min. after the completion of injection) as follows:

$$X_3=0.88v\int_{t_4}^{t_5} cdt \quad \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

By plotting the relation of time and the PAH concentration in the circulating plasma as in Fig. 1, we may calculate the approximate value of $\int_{t_4}^{t_5} cdt$, and as v may be calculated by the method described in our previous report[1], X_2 and X_3 can be calculated from the equations (5) and (6) above. When the total PAH quantity (U_1) contained in the urine up to the point of time t_5 is measured, the PAH quantity (X_1) lost into the urine between the point of time 0 (when the injection was initiated) and t_1 is of course as follows:

$$X_1=U_1-(X_2+X_3) \quad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

For the time range $0-t_o$, when the point of time of completion of injection is expressed by t_o, the PAH quantity in the circulating plasma Z may be represented as follows, provided that the injection is completed under a constant speed:

$$Z=\frac{2000t}{t_o}-(x+y) \quad \ldots\ldots\ldots\ldots\ldots\ldots (8)$$

(x and y stand for the PAH quantity carried away into the urine and tissues from the circulating plasma respectively.)

Considering that, in the time range t_1-t_4, the PAH quantity given off into the tissues as well as into the urine from the circulating plasma was found to stand in direct ratio to the PAH concentration in the circulating plasma, as described in the foregoing, it may be safely presumed that in the time range $0-t_o$ too, the same relation applies, inasmuch as an exces-

sive speed in carrying out the injection does not bring about the quick increase in the PAH concentration in the circulating plasma. This assumption leads to the following equation:

$$dy=\delta\ cdt \quad \cdots\cdots (9)$$

wherein δ represents a constant.

Therefore

$$x+y=(0.88v+\delta)\int cdt=\frac{0.88v+\delta}{V_p}\int Z\ dt$$

and therefore (8) may be rewritten as follows:

$$Z=\frac{2000}{t_o}t-\frac{0.88v+\delta}{V_p}\int Z\ dt$$

$$\therefore\quad \frac{dZ}{dt}=\frac{2000}{t_o}-\frac{0.88v+\delta}{V_p}Z \quad \cdots\cdots (10)$$

Solving this differential equation, we get

$$\frac{1}{\gamma}\log_{10}\left(\frac{2000}{t_o}-\frac{\gamma}{0.4343}Z\right)=-t+C \quad \cdots\cdots (11)$$

($\gamma=\frac{(0.88v+\delta)\ \log_{10}e}{V_p}$ and C is a constant of integration)

As Z=0, when t=0

$$C=\frac{1}{\gamma}\log_{10}\frac{2000}{t_o}$$

Then from (11), we come to

$$\log_{10}\left(\frac{2000}{t_o}-\frac{\gamma}{0.4343}Z\right)=\log_{10}\frac{2000}{t_o}-\gamma t \quad \cdots (12)$$

If we presume, as is probable, that the PAH quantity lost into the urine and tissues in the time range t_o-t_1, similar to that in time range $0-t_o$, stands in a direct ratio to the PAH concentration in the circulating plasma, and if we express the PAH quantity carried away into the urine and tissues by x and y respectively, the PAH quantity decreased in the circulating plasma by z, the PAH concentration in the circulating plasma by c and the PAH quantity present in the circulating plasma at the point of time t_1 by a, the following may be derived:

$$z=x+y$$

$$\therefore\quad dz=dx+dy=(0.88v+\delta)\ cdt=\frac{0.88v+\delta}{V_p}(a-z)\ dt$$

Solving this equation, we get the result:

$$\log_{10}c=-\gamma t+C \quad \cdots\cdots (13)$$

(C is a constant of integration)

When $t=t_1$, $c=c_1$. Therefore

$$C=\log_{10} c_1+\gamma t_1$$

and as $t_1=5$, (13) is rewritten as follows:

$$\log_{10} c=\log_{10} c_1+(5-t)\gamma$$
$$\therefore\ c=10^{(5-t)\gamma}\cdot c_1$$

When $c=c_o$ at the point of time t_o, we get

$$c_o=10^{5\gamma}\cdot c_1 \quad \ldots\ldots (14)$$

As the PAH quantity in the circulating plasma at the point of time t_o is represented by $c_oV_p=10^{5\gamma}\cdot c_1V_p$, from (12) we derive

$$\log_{10}\left(\frac{2000}{t_o}-\frac{10^{5\gamma}c_1\gamma V_p}{0{,}4343}\right)=\log_{10}\frac{2000}{t_o}-\gamma t_o \quad \ldots\ldots (15)$$

Let X_1 and Y_1 stand for the PAH quantity in the urine and tissues at the point of time t_1, and the quantity of injected PAH be 2,000 mg., then

$$X_1+Y_1+c_1V_p=2000 \quad \ldots\ldots (16)$$

and

$$X_1+Y_1=(0.88v+\delta)\int_o^{t_1} c\ dt$$

Whereas $X_1=0.88v\int_o^{t_1} c\ dt$, therefore

$$X_1+Y_1=\frac{X_1}{0.88v}(0.88v+\delta)=\frac{\gamma X_1 V_p}{0.38v} \quad \ldots\ldots (17)$$

From (16) and (17), we get

$$\left(\frac{\gamma X_1}{0.38v}+c_1\right)V_p=2000 \quad \ldots\ldots (18)$$

From the above two equations (15) and (18), we can calculate the values of γ and V_p, the latter representing the circulating plasma volume, and from this and the hematocrit value the circulating blood volume can be calculated.

Method and Results of Experiments

The method for estimating the renal plasma flow v is as described in our previous report[1], and as previously reported two values of v were calculated for the time ranges 30–50 min. and 50–70 min. after the injection of PAH, but in this report, the former value was used in preference, since the former time range represents a status somewhat nearer in time to the time range $0-t_s$ treated in this report.

The injections of PAH, 2,000 mg. per dosis, were completed at a constant speed in 2–3 min. (in most cases in 2½ min.), and besides the

sampling of blood and urine described in our previous report[1], blood samples were taken at the points of time 5, 10, 15 and 20 min. after the injection for measuring the PAH concentration in the circulating plasma. In these experiments, instead of giving the subjects 500–700 cc. of water to drink prior to the experiments, as in the previous experiments, water was given from time to time during the experiments, so as to equalize the urination as far as possible. A Duboscq's colorimeter was used instead of the Pulfrich's photometer used in the former experiemts. In other points, the same methods of experimentation were followed.

TABLE I

Values of γ and Circulating Plasma Volume

No.	Sex	Age	γ	Circulating plasma volume (c.c.)	
				Vp	Calculated by previous method
1	♂	38	0.071	4835	4020
2	♂	31	0.112	3546	3839
3	♂	42	0.112	3590	2891
4	♂	32	0.117	2959	3055
5	♂	24	0.088	3295	2833
6	♂	30	0.102	4048	4302
7	♂	29	0.141	2454	3403
8	♂	27	0.095	3862	4192

In most cases, male subjects in normal health were chosen for the experiment, and the values of V_p, obtained from 8 subjects coming up to the good conditions for experiments by the application of the above expounded theory, together with the values of the circulating plasma volume obtained in accordance with the method described in the previous report as well as the values for γ are listed in Tab. 1. For calculating the values of V_p by equations (15) and (18), the values of V_p obtained by (18) were substituted for V_p in (15), arriving at the following equation:

$$1-10^{-t_0\gamma}=0.88c_1\ t_0\frac{10^{5\gamma}}{\frac{X_1}{V}+\frac{0.38c_1}{\gamma}}$$

From this, the values for γ were deduced by the help of a logarithmic table. Using the values for γ obtained thus, the values for V_p were calculated by (18).

Upon comparing the circulating plasma volume calculated by this new method and the method reported previously, the values according to both the methods show approximate agreement in all cases except in cases

No. 1, No. 3 and No. 7, where the discrepancy is rather remarkable. As mentioned above, the previous method was built upon the assumption that the PAH quantity in the circulating plasma in a normal person represents 15.3% of the total PAH in the body 30 min. after the injection, but this percentage is a mean value of many measurements, so that it is quite natural that the values of the circulating plasma volume by the previous method do not always coincide exactly with the real value, and so it is also natural, that the former values do not always coincide exactly with the values calculated by this new method. As case No. 1 was suffering from severe anemia due to chronic hemorrhage accompanied by a gastric ulcer and case No. 3 also had moderate anemia, it is quite reasonable that in these cases the values obtained by this new method proved larger than those calculated by the previous method. It is a natural consequence of the theory underlying the previous method.

The above results of the experiments seem to support my theory outlined in the above and to justify my expectation that the circulating plasma volume, and consequently, the circulating blood volume may be computed in any case, were it in a normal subject or in a pathological case as well. It may be imperative, however, to take special care in choosing the right moment for taking samples of blood and urine, when we wish to apply this new method to cases suffering from advanced edema.

References

1) Kishine *et al.*, Tohoku J. Exp. Med., 1952, **56**, 275.

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Application of Radioisotope P^{32} for Treatment of Incurable Latent Syphilis

Preliminary Report

By

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The therapy of syphilis has advanced by leaps and bounds since penicillin came into use for its treatment. However, the rate of recovery of latent syphilis cases has been low, variously reported as 48.4%,[1] 68.6% (in cases of incomplete previous treatment or 72.4% (in cases without previous treatment)[2] and 28.9%[3] in early syphilis cases, and 47% (cases of unknown duration of disease),[4] 55.43% (in cases of incomplete previous treatment) or 49.14% (in cases without previous treatment)[2] and 18.2%[3] in late syphilis.

According to the reports of past researchers,[5][6] we are taught that the treponema pallidum responsible for latent syphilis are mainly distributed in the liver, the spleen and the lymph system in the patients. Also it has been reported[7][8] that P^{32} prefer entially is distributed in the same tissues. Accordingly, if P^{32} is administered to latent syphilis patients, may we not anticipate that the treponema pallidum colonies nestled in these tissues be deranged and thereby the action of the antiluetica be promoted? For the purpose of verifying such an anticipation, the following experiments were carried out.

Experimental

I. Clinical Experiment

1. *Materials:* As shown in Table I, six latent syphilis patients who refused to show negative sero-reaction after repeated antisyphilitic therapy were chosen as subjects, including two congenital syphilis, one late syphilis and three early syphilis cases. As reagent, $H_3P^{32}O_4$ solution containing 5 mc. of P^{32} (15A-P in the catalogue of AEC) was diluted with about 20 cc. of distilled water to a concentration of 0.25 mc. per 1.0 cc., poured into ampoules and disinfected by boiling.

TABLE I

History of Patients before P^{32} Administration

Diag.	Name	Age	Sex	Infection		Months before P^{32} administration: 31 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
Early syphilis	KM	23	f	two years ago	therapy	↓p 24 m.u. ↓p 6 m.u. ↓p 6 m.u.
					WaR	### ### ### + + ## ## + + + + ## + ## +
Late syphilis	MM	33	m	ten years ago	therapy	↓p 24 m.u. ↓p 6 m.u. ↓p 9 m.u.
					WaR	### ### ### ### ## + + + + + ## ## ## + + + + ## ## ##
Congenital syphilis	IM	3	m		therapy	↓p 15 m.u. ↓p 3 m.u. ↓p 6 m.u. ↓p 3 m.u.
					WaR	### ### + ## + + ### + + ± – ## – + + +
Early syphilis	EU	28	m	thrre years ago	therapy	↓ m & Bi ↓N-A ↓p 12 m.u. N-A & Bi ↓p 12 m.u.
					WaR	+ + ± – – – – + – – – – + – + +
Early syphilis	TK	26	m	two years ago	therapy	↓p 9 m.u. ↓ com. ↓p 9 m.u. m & Bi
					WaR	### ### ## ### + + + ## ## + + + +
Congenital syphilis	KO	20	f		therapy	↓p 6 m.u. ↓p 9 m.u. m & Bi com.
					WaR	## ## + ## ## ## ## ## ##

NB. p: penicillin, m: mapharsen, N-A: neoarsenobenzol (V.P.J.), Bi: bismuth. salicyl. com: communin (Shwartzman filtrate), m.u.: million units.

2. *Method:* 0.5 mc. of P^{32} each (half of the doses to an infant patient of congenital syphilis) was administered per case intravenously. Excepting three cawes, one case each of congenital, late and early syphilis who were used as controls, the remaining three cases were subjected to penicillin therapy beginning about 10 days after the P^{32} injection. Observation was kept up for nearly one month after the injection, not only their sero-reaction, but also urobilinogen and urobilin in their urine, their hematologic findings (including serum protein level, specific gravity of serum, sedimentation rate of erythrocytes etc.) and the pH values of their saliva were tested by routine method, and the radio-activity of their peripheral blood extracted from their earlobes, of their venous blood, urine and feces was measured by means of a Geiger-Müller radiation counter. Their serum-reaction was followed up for several months thereafter, tested once every month.

3. *Results:* i) Cases injected with P^{32} alone. By WaR tests, all the cases, including one congenital syphilis case, male, three years old, one late syphilis case, male, 33 years old and one early syphilis case, married female, 23 years old, showed unmistakable fluctuation of sero-reaction. In particular, the late syphilis case complained of pain at the distal end of the right humerous and the proximal end of the right tibia and a feeling of distension of the epigastrium, in the very night following the injection. Upon examination on the next day, the liver was found swollen above the right mammillary line by two fingers' breadth and feeling pain upon pressure. That night, he began to feel stricture of the pharynx and hoarseness, as well as weakening of sight. The pain at the liver and the long bones disappeared in five days, followed by the relief from the closeness of the pharynx and the hoarseness, but the enlargement of the liver took about half a month to abate (Table II).

The hematologic findings and the results of the other routine clinical examinations showed more or less fluctuations, but no case showed any marked deviation above physiologically normal ranges. The radioactivity of the earlobe blood of the late syphilis patient became inappreciable after 18 days, but in all other samples of blood, urine and feces, it diminished daily, but remained detectable even about one month after the injection (Table III).

ii) Cases treated with penicillin after P^{32} injection. One of the tested two early syphilis cases became negatively reactive in WaR test upon injection, but the other cases, consisting of one early and one congenital syphilis cases, showed no change in serum WaR. The three cases were injected each with 9,000,000 units of procaine penicillin G in total, in 15 doses of 600,000 units per injection, administered every second days starting from around the 11th day after the P^{32} injection. The case who

TABLE II

Changes of Sero-reaction after P^{32} Administration with or without Penicillin Therapy

Diag.	Name	Age	Sex	Infection		Day (after P^{32} administration) 1	2	3	4	5	6	8	11	12	13	14	15	16	18	19	21	– – – –	Month (″) 1	2	3	4	5
Early syphilis	KM	23	f	two years ago	therapy																						
					WaR	⧺	⧺		+	+		+	⧺						+				⧺	+	+	+	
Late syphilis	MM	33	m	ten years ago	therapy																						
					WaR	+	+		+	±		+	+						±				+	⧺	+	+	
Congenital syphilis	IM	3	m		therapy																						
					WaR	+	+		±	±		+	±						±				+	+	⧺	±	
Early syphilis	EU	28	m	three years ago	therapy									←	penicillin 9 million units							→					
					WaR	–	±		–	–		–	–										–	–	–	±	–
Early syphilis	TK	26	m	two years ago	therapy									←	penicillin 9 million units							→					
					WaR	+							+										+	±		+	
Congenital syphilis	KO	20	f		therapy							↓ P^{32} 0.5 mc	←	penicilline 9 million units								→					
					WaR	⧺	⧺		⧺		⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺		⧺	⧺	⧺		

turned WaR negative immediately upon P^{32} injection remained negative in the 5th month after the injection, but the two other cases showed no change in their positive reaction down to the 4th month following the treatment (Table II). The observations on hematologic findings and radioactivity showed similar results as those by the cases injected with P^{32} alone described in i) above.

II. Experiments with Animals

1. *Materials*: 11 rabbits of about three kg. each in body weight inoculated with syphilis germs in their testicles with positive WaR, were used 100 days to 12 months after the inoculation. Four of them were administered procaine penicillin G, three others were injected with P^{32} and the remaining four were subjected to penicillin treatment thereafter, and the effect of such treatments on the serum reaction was observed in the three groups. The Nichols' strain and Kojimachi strain of rabbit syphilis germs donated by Dr. Shiro Someya of the Institute of Public Health, Tokyo, were used for inoculation, at which operation the kind suggestion of Mr. Rin Awano of the same Institute proved of great value. We hereby express our gratitude due to both of them.

The same grade of P^{32} used with human subjects mentioned above was used in the following experiments.

2. *Method*: The control group was injected with approximately 70,000 units per kg. of body weight of procaine penicillin G intragluteally. P^{32} was administered in a dose of 0.04 mc. per kg. of body weight per test by injection into the auricular veins. When both the reagents were applied, penicillin was administered as described above 48 hrs. after the injection of P^{32}. In every case, sero-reaction was kept under observation for 65 days thereafter.

3. *Results:* i) Effect of penicillin injection on the sero-reaction of syphilized rabbits (Table IV). With the rabbit No. 8, treated seven months after the inoculation with Nichols' strain, the reaction turned negative once at the outset but relapsed positive in a short time, to gain permanent negativity 60 days afterwards. Another rabbits No. 10 treated five months after the inoculation turned negative 60 days after the penicillin injection in Murata reaction alone, with no change in WaR throughout. No. 9, treated five months after the inoculation with Kojimachi strain, showed fluctuation in both Murata reaction and WaR, but died on the 11th day. No. 11, treated three months after the inoculation of Nichols' strain also showed such fluctuations and died on the 13th day of experiment.

ii) Effect of P^{32} on ditto (Table V). The rabbit No. 5, injected with P^{32} 12 months after the inoculation with Kojimachi strain, showed

TABLE

Radioactivity in some Materials

(Count per min. on Geiger-

Diag.	Name	Age	Sex	Materials		Day 1	2	4	5
Early syphilis	KM	23	f	peripheral blood	0.01 cc	31.35	17.4	10.2	9.2
				venous blood	0.1 cc	112.8	48.4	39.4	39.4
				urine	0.1 cc	655.7	302.7	216.4	145.8
				feces	0.1 g	578.4	223.4	113.7	108.8
Late syphilis	MM	33	m	peripheral blood	0.01 cc	17.4	10.85	9.5	8.85
				venous blood	0.1 cc	295.8	59.7	45.4	39.4
				urine	0.1 cc	555.3	496.7	263.7	227.4
				feces	0.1 g	281.4	224.4	211.7	169.8
Congenital syphilis	IM	3	m	peripheral blood	0.01 cc	33.7	20.9	16.7	14.2
				venous blood	0.1 cc	150.4	91.8	71.7	57.4
				urine	0.1 cc	758.7	377.9	295.4	232.4
				feces	0.1 g	246.7	240.4	201.8	167.4
Early syphilis	EU	28	m	peripheral blood	0.01 cc	48.9	8.0	3.7	3.35
				venous blood	0.1 cc	177.7	120.8	51.7	38
				urine	0.1 cc	9918.8	635.4	251.7	145.4
				feces	0.1 g	240.4	120.8	92.2	73
Early syphilis	TK	26	m	peripheral blood	0.01 cc	16.7			
				veneous blood	0.1 cc	64.4			
				urine	0.1 cc	447.4			
				feces	0.1 g	147.4			
Congenital syphilis	KO	20	f	peripheral blood	0.01 cc	55.2	30.7	13.7	
				venous blood	0.1 cc	180.4	161	104.7	
				urine	0.1 cc	1540.4	248.7	201.8	
				feces	0.1 g			595.4	

negative sero-reaction from the 4th day but on the 11th day, the reaction began to rise stronger than ever. No. 6 and 7, treated some eight months after the inoculation with Nichols' strain, showed intensified positive reaction from the 3rd and 5th days respectively. The latter two died on the 9th and 12th days.

iii) Fluctuation of sero-reaction upon P^{32} administration followed by penicillin therapy (Table VI). The rabbit No. 1, treated with P^{32} eight months after the inoculation with Kojimachi strain, immediately showed a rise in the sero-reaction, but upon injection of penicillin 48 hrs. afterwards, the reaction became negative, intensified on the 7th day following the P^{32} injection or the 4th day after penicillin administration, and dropped again to negativity 60 days afterwards for good. No. 2 and 3, treated three months after the inoculation with Kojimachi and Nichols' strains respectively, showed no change in sero-reaction upon injection either of P^{32} or of penicillin. The former died in 18 days, while the latter ives and is under observation, now 65 days after the first treatment. No.

III

after P^{32} Administration

Müller's radiation counter)

6	8	11	12	13	14	15	16	18	20	25	32	39
	6.85	4.15						1.4		1.2		
	20.7	15.7						3.8		0.3		
	143.4	91.4						38.7		9.9	1.8	
	57.7	44.0						37.4		11.8	6.7	
	4.2	2.65						0		0		
	35.7	14.3						10.8		3.2		
	184.4	98.4						83.4		5.3		
	153.4	96.0						83.4		8.3	5.3	
	5.35	2.2						0.9		0.25		
	49.4	49						34.9		7.3		
	221.8	181.4						164.7		90.4	32.8	28.6
	48.4	39.4						12.9		5.3		
	1.7	0.7										
	37.4	9.4										
	140.7	90.4		60.4								
	19.4											
		10.35										
		59.4										
5.2	3.65	141.7	45.4	14.2	8.85	4.7						
44.4	18.3	1815.7	176.4	141.4	74.4	25.4						
175	125.4	2571.4	621.4	209.4	177.7	162.4	124.8	117.7	38.4			
	20.4		848.4			5.73						

4, treated three months after the inoculation with Nichols' strain showed fluctuations in sero-reaction following injections of both P^{32} and penicillin, but died in 15 days.

Summary and Discussion

Noticing that the tissues where treponema pallidum is observed in existence in latent syphilis cases and where P^{32} is found precipitated with preference are about the same, we undertook the above experiments for promoting the efficiency of antiluetica by taking advantage of derangement occasioned by P^{32} in the colonies of syphilis germs entrenched in those tissues in incurable latent cases. In the experiments we assumed that the resistance in sero-reaction indicated persistence of syphilitic condition. The results obtained may be summarized as follows. Upon administration of P^{32} to latent syphilis patients in various stadia showing resistance against negation of sero-reaction by means of any available antiluetic therapy, it

TABLE IV

Changes of Sero-reaction after Penicillin Administration in Rabbits

Rabbits No.	Inoculation of Treponema pallidum		Penicillin administration			Sero-reaction before and after penicillin administration											
	strain	date	date	body weight (kg)	dosis (mil. unit)	kinds of reaction	before	after (day) 1	2	3	5	6	9	11	30	40	60	65
8	Nichols	July 30 1952	March 4 1953	2.05	0.2	Wa.R.	±±	−	−	±±	±±	±±	±±	±±	±±	±±	−	−
						Murata-R	+	−	−	++	++	++	++	++	−	−	−	−
						Ogata-R	++	++	−	±±±	±±±	±±±	±±±	±±±	±±±	±±±	−	−
						Slide-T	−	−	−	±±±	±±±	±±±	±±±	±±±	±±±	±±±	−	−
9	Kojimachi	Oct. 2 1952	"	3.15	0.2	Wa.R.	±±	++	++	++	++	++	++	++	*			
						Murata-R	+	−	−	−	+	+	+	+				
						Ogata-R		++	++	++	±±	±±	±±	±±				
						Slide-T		±±	+	+	+	++	++	++				
10	Nichols	"	"	2.80	0.2	Wa.R.	±±	±±	±±	±±	±±	±±	±±	±±	±±	±±	±±	±±
						Murata-R	−	−	−	±±±	±±±	±±	±±	±±	±±	±±	−	−
						Ogata-R		±±±	±±±	±±±	±±±	±±±	±±±	±±±	±±±	±±±	±±±	±±±
						Slide-T		±±±	±±±	±±±	±±±	±±±	±±±	±±±	±±±	±±±	±±±	±±±
11	Nichols	Dec. 8 1952	"	2.93	0.2	Wa.R.	±±	++	++	++	++	++	++	++	**			
						Murata-R	++	−	+	+	++	++	++	++				
						Ogata-R		±±	±±	±±	±±	±±	±±	±±				
						Slide-T		±±	±±	±±	±±	±±	±±	±±				

NB. Wa.R. : Browning's method of complement fixation reaction, Murata-R : Murata's method of precipitation reaction, Ogata-R. : Ogata's method of complement fixation reaction, Slide-T : Ogata's modification of VDRL, * Died on the 11th day after penicillin administration, ** Died on the 13th day after penicillin administration.

TABLE V

Changes of Sero-reaction after P^{32} Administration in Rabbits

Rabbits No.	Inoculation of Treponema Pallidum		P^{32} administration			Sero-reaction before and after P^{32} administration												
	Strain	date	date	body weight (kg)	dosis (mc)	kinds of reaction	before	After (day) 1	2	3	4	5	7	11	13	30	40	60	65
5	Kojimachi	Feb. 15 1952	March 2 1953	2.80	0.112	Wa.R.	++	+	+	+	−	−	−	###	###	###	###	###	###
						Murata-R	−	+	+	+	−	−	−	###	###	###	###	###	###
						Ogata-R		###	###	###	−	−	−	###	###	###	###	###	###
						Slide-T		++	++	++	−	−	−	###	###	###	###	###	###
6	Nichols	July 16 1952	"	2.72	0.09	Wa.R.	±	+	+	++	++	++	++	++	*				
						Murata-R	−	−	−	###	###	###	###	###					
						Ogata-R		###	###	###	###	###	###	###					
						Slide-T		###	###	###	###	###	###	###					
7	Nichols	July 30 1952	"	3.05	0.12	Wa.R.	###	++	++	++	++	###	###	**					
						Murata-R	+	−	−	−	−	###	###						
						Ogata-R		++	++	++	++	###	###						
						Slide-T		###	###	###	###	###	###						

NB. Wa. R.: Browning's method of complement fixation reaction, Murata-R: Murata's method of precipitation reaction, Ogata-R: Ogata's method of complement fixation reaction, Slide-T.: Ogata's modification of VDRL, * Died on the 12th day after P^{32} administration, ** Died on the 9th day after P^{32} administration.

TABLE VI

Changes of Sero-reaction after P^{32} Penicillin and Administration in Rabbits

Rabbits No.	Inoculation of treponema pallidum		P^{32} administration			Soro-reaction before and after P^{32} administration			Penicillin administration (dosis: mil. units)	Sero-reaction							
	strain	date	date	body weight (kg)	dosis (mc)	kinds of reaction	before	After (day) 1	2		after successive days 3	4	7	9	30	40	60	65
1	Kojimachi	July 16 1952	March 2 1953	2.50	0.075	Wa.R.	+	++	++	0.18	−	−	##	##	##	##	−	−
						Murata-R	−	−	−		−	−	++	++	−	−	−	−
						Ogata-R		###	###		−	−	++	###	###	++	−	−
						Slide-T		++	++		−	−	##	###	##	+	−	−
2	Kojimachi	Dec. 8 1952	"	3.70	0.124	Wa.R.	##	##	##	0.25	##	##	##	##	*			
						Murata-R	##	##	##		##	##	##	##				
						Ogata-R		###	###		###	###	###	###				
						Slide-T		###	###		###	###	###	###				
3	Nichols	Dec. 8 1952	"	3.50	0.101	Wa.R.	##	##	##	0.25	##	##	##	##	##	##	##	##
						Murata-R	##	##	##		##	##	++	++	++	++	++	++
						Ogata-R		###	###		###	###	###	###	###	###	###	###
						Slide-T		###	###		###	###	###	###	###	###	###	###
4	Nichols	Dec. 8 1952	"	2.40	0.073	Wa.R.	±	−	−	0.16	−	−	##	##	**			
						Murata-R	−	−	##		−	−	##	##				
						Ogata-R		++	−		−	−	###	###				
						Slide-T		+	−		−	−	###	###				

NB. Wa. R.: Browning's method of complement fixation reaction, Murata-R: Murata's method of precipitation reaction, Ogata-R: Ogata's method of complement fixation reaction, Slide-T: Ogata's modification of VDRL, * Died on the 185h day after P^{32} administration, ** Died on the 15th day after P^{32} administration.

was found that a dose of 0.5 mc. of P^{32} could not effect any changes in WaR of a female congenital syphilis case, 20 years of age, and of an early syphilis case of 26 years old man, but the other four, early, late and congenital syphilis cases thus treated showed frank fluctuations of WaR. Two early and one congenital syphilis cases, including the two former with no change in sero-reaction, were given additional treatments with nine million units of penicillin each starting from around 10 days after the P^{32} injection. One of them turned negative in his WaR and the subsequent penicillin treatment succeeded in keeping the negative in his WaR five months afterwards, but the other two cases failed to respond, either to P^{32} or to the penicillin therapy.

However, we must note that one late syphilis case began to complain of pain at the ends of the long bones of the extremities, enlargement of the liver with tenderness, noarseness and weakening of visual faculty upon injection of P^{32}. This fact, together with the fluctuations in WaR observed by four of the six tested cases, seems to throw a ray of hope in the realization of the intended derangement of treponema pallidum.

All the three cases of syphilized rabbits injected with P^{32} solution showed decided fluctuations in their sero-reaction, two of them dying in 9 and 12 days respectively. Also all the four cases with P^{32} injection followed up with penicillin administration showed similar fluctuation and two of them died in 15 and 18 days respectively. Our observation of 65 days, however, revealed permanent negative WaR only in one case out of 11 tested.

In any case, one thing seems to be undisputable, namely, that, in both the latent syphilis cases and the syphilized rabbits, the injection of P^{32} occasioned fluctuations of sero-reaction, and we may be justified in assuming that this effect was due to the derangement of treponema pallidum by the biotropism of the radioactive P^{32}. We regret to state that we were restricted in our experimental activities on human and animal subjects owing to the strict limitation in time available for the procurement of P^{32}, so that we are herewith forced to give out a preliminary report on our past experiments, though we are far from being satisfied with our rather cursory works.

We hereby express our heartfelt gratitude due to Prof. Nobutane Mori and all the staff members of the Physiological Laboratory of this University for their valuable assistance and suggestions, which enabled us to accomplish the parts in our experiments connected with handling of radioactive matters. It must be also mentioned with thanks that the cost of these experiemnts were defrayed from the Fund for Scientific Research allocated by the Education Ministry.

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Biophysikalische Studien über Blutserum-Eiweisse

III. Ueber das Verhalten einiger Mineralstoffe im Blutserum bei elektrischer Aufladung*

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Einleitung

In unseren vorigen Arbeiten[1)-2)] haben wir gezeigt, dass die Flockungszahl (Flz) des Blutserums bei der „ positiven " Aufladung gesunder Personen (z.B. bis zu +450 V) proportional der Spannung stets erheblich ansteigt, während sie bei der „ negativen " Aufladung (z.B. bis zu –450 V) mit abnehmender Spannung imemr deutlich abfällt. Diese charakteristische, elektrobiologische Erscheinung ist besonders signifikant ausgeprägt bei der Flockungszahlreaktion nach der von Murasugi modifizierten Methode, welche auf den Reaktionsausfall speziell der „ T-Franktion " (Takata), einer neuen Komponente der Serumproteine, hinzielt. Diese Beobachtungen legten es sehr nahe, festzustellen, ob auch Mineralstoffe im Blutserum bei der elektrischen Aufladung, ähnlich wie die Flockungszahl, eine gesetzmässige Verschiebung aufweisen können. Um diese Frage zu klären, haben wir Untersuchungen über das Verhalten einiger Mineralstoffe, und zwar von Natrium, Kalium und Calcium, bei der positiven bzw. negativen Aufladung vorgenommen.

Experimentelles

Zur Bestimmung des Natrium haben wir die Methode nach Kramer-Gittleman[3)], des Kalium nach Kramer-Tisdall[4)] und des Calcium das Verfahren nach Clark-Collip[5)] verwendet. Die Untersuchungen wurden hier ausschliesslich nur an gesunden Personen gemacht. Die Versuchsergebnisse in vivo sind in Tabelle I und II anschaulich wiedergegeben:

Wie Tabellen I und II zeigen, verschieben sich die Kationen bei der elektrischen Aufladung des Menschen immer regelmässig nach einer be-

* Die vorliegende Arbeit wurde auch unter Leitung von Prof. Dr. Maki Takata durchgeführt.

stimmten Richtung. Bei der „ positiven " Aufladung (z.B.+450 V) steigt

TABELLE I

Verhalten einiger Mineralstoffe im Serum bei der positiven Aufladung des Menschen (Versuche in vivo)

Datum und Name		Vor Aufladung	+450 V (15 Min.)	10 Min. nach beendeter Aufladung	30 Min. nach beendeter Aufladung	Maximal-Differenz
20.5.52	Na (mg%)	294	283	—	288	−11
S. Takeuchi	K (mg%)	24.9	26.2	—	25.8	+1.3
♀ 34 J.	Ca (mg%)	11.6	11.2	—	11.2	−0.4
24.5.52	Na (mg%)	330	320	—	329	−10
Y. Miyamoto	K (mg%)	16.4	17.6	—	17.2	+1.2
♂ 26 J.	Ca (mg%)	11.2	10.4	—	10.4	−0.8
27.5.52	Na (mg%)	332	327	329	331	− 5
K. Kishimoto	K (mg%)	16.9	18.0	18.0	17.7	+1.1
♀ 25 J.	Ca (mg%)	11.6	11.2	11.2	11.6	−0.4
30.5.52	Na (mg%)	353	338	338	348	−15
T. Tsutsui	K (mg%)	16.4	18.5	18.2	17.6	+2.1
♀ 35 J.	Ca (mg%)	12.4	12.0	12.4	12.4	−0.4
4.6.52	Na (mg%)	352	345	346	349	− 7
H. Yamada	K (mg%)	15.4	15.8	15.8	15.6	+0.4
♂ 34 J.	Ca (mg%)	12.4	12.0	12.0	12.4	−0.4
6.6.52	Na (mg%)	353	349	350	352	− 4
S. Matsui	K (mg%)	15.9	16.3	16.2	16.2	+0.4
♂ 24 J.	Ca (mg%)	11.2	10.8	11.2	11.2	−0.4
14.6.52	Na (mg%)	350	347	348	349	− 3
M. Fujii	K (mg%)	16.6	18.0	17.8	17.5	+1.4
♂ 30 J.	Ca (mg%)	11.6	11.2	11.2	11.2	−0.4
3.7.52	Na (mg%)	355	335	340	353	−20
T. Fujii	K (mg%)	15.7	16.7	16.6	16.4	+1.0
♀ 28 J.	Ca (mg%)	11.2	10.8	11.2	11.2	−0.4
11.7.52	Na (mg%)	348	335	338	343	−13
R. Igaki	K (mg%)	17.2	17.5	17.2	17.2	+0.3
♂ 24 J.	Ca (mg%)	10.8	10.8	10.8	10.8	±0
21.7.52	Na (mg%)	301	276	285	301	−25
M. Yadagawa	K (mg%)	19.5	19.8	19.8	19.4	+0.4
♂ 29 J.	Ca (mg%)	10.8	10.2	10.8	10.8	−0.6
20.8.52	Na (mg%)	327	324	325	325	− 3
G. Fujii	K (mg%)	20.2	21.9	21.6	21.0	+1.7
♂ 51 J.	Ca (mg%)	10.0	9.2	9.2	9.6	−0.8
24.8.52	Na (mg%)	342	334	334	338	− 8
G. Sugita	K (mg%)	20.6	21.1	21.1	20.9	+0.5
♂ 23 J.	Ca (mg%)	11.2	10.8	10.8	11.0	−0.4
28.8.52	Na (mg%)	363	355	361	363	− 8
T. Yoshida	K (mg%)	21.6	22.3	21.8	21.1	+0.7
♂ 23 J.	Ca (mg%)	12.4	12.0	12.0	12.2	−0.4

2.9.52	Na (mg%)	337	328	328	332	− 9
S. Yoshida	K (mg%)	21.2	22.5	22.1	21.5	+1.3
♂ 25 J.	Ca (mg%)	12.8	12.4	12.4	12.4	−0.4
4.9.52	Na (mg%)	371	355	361	369	−16
S. Kakiuchi	K (mg%)	20.9	21.3	21.2	20.7	+0.4
♂ 26 J.	Ca (mg%)	11.2	10.8	10.8	11.2	−0.4
15.9.52	Na (mg%)	378	367	371	375	−11
K. Fujii	K (mg%)	24.4	25.8	25.8	25.7	+1.4
♂ 26 J.	Ca (mg%)	12.0	11.2	11.2	11.2	−0.8
25.9.52	Na (mg%)	348	344	346	347	− 4
K. Kishimoto	K (mg%)	17.8	19.5	17.7	17.8	+1.7
♂ 25 J.	Ca (mg%)	11.6	11.2	11.6	11.6	−0.4
8.10.52	Na (mg%)	367	359	359	364	− 8
R. Igaki	K (mg%)	17.7	19.4	18.8	18.7	+1.7
♀ 24 J.	Ca (mg%)	—	—	—	—	—
10.10.52	Na (mg%)	347	344	346	347	− 3
T. Fujii	K (mg%)	21.8	23.8	23.6	23.4	+2.0
♀ 28 J.	Ca (mg%)	10.8	10.0	10.4	10.4	−0.8
	Na (mg%)	345	355			−10
Durchschnitt	K (mg%)	19.2	20.1			+0.9
	Ca (mg%)	11.5	11.0			−0.5

Tabelle II

Verhalten einiger Mineralstoffe im Serum bei der negativen Aufladung des Menschen (Versuche in vivo)

Datum und Name		Vor Aufladung	−450 V (15 Min.)	10 Min. nach beendeter Aufladung	30 Min. nach beendeter Aufladung	Maximal-Differenz
19.5.52	Na (mg%)	—	—	—	—	—
S. Yasukawa	K (mg%)	26.2	25.6	—	25.8	−0.6
♂ 36 J.	Ca (mg%)	11.2	12.0	—	11.2	+0.8
14.6.52	Na (mg%)	351	368	362	353	+17
M. Fujii	K (mg%)	16.8	16.4	16.4	16.6	−0.4
♂ 30 J.	Ca (mg%)	12.0	12.4	12.0	12.0	+0.4
16.7.52	Na (mg%)	356	376	373	358	+20
K. Fujii	K (mg%)	20.3	19.7	19.3	19.3	−0.6
♂ 26 J.	Ca (mg%)	10.4	10.8	10.8	10.4	+0.4
12.9.52	Na (mg%)	383	385	384	383	+ 2
R. Hasei	K (mg%)	19.7	17.3	17.3	18.2	−2.4
♂ 24 J.	Ca (mg%)	12.0	12.8	12.0	12.0	+0.8
1.10.52	Na (mg%)	378	384	383	383	+ 6
S. Takeuchi	K (mg%)	24.4	24.2	24.4	24.4	−0.2
♀ 34 J.	Ca (mg%)	11.2	11.4	11.2	11.2	+0.2
	Na (mg%)	367	378			+11
Durchschnitt	K (mg%)	21.5	20.6			−0.9
	Ca (mg%)	11.4	11.9			+0.5

der Kalium-Spiegel sofort an, während der Gehalt an Natrium und Calcium gewissermassen abfällt. Bei der „negativen" Aufladung der Versuchspersonen (z.B. −450 V)sind die Beziehungen aber ganz umgekehrt. So fällt der Kalium-Spiegel dabei ab, während der Gehalt an Natrium und Calcium nicht unwesentlich ansteigt. Nach beendeter Aufladung kehren die veränderten Kationen-Werte so schnell, und zwar innerhalb von 30 Minuten, schon wieder zu Ausgangswerten zurück oder sie werden fast normalisiert.

Es musste daher weiterhin festgestellt werden, ob nicht eine gesetzmässige Verschiebung der Kationen bei der elektrischen Aufladung des Menschen lediglich auf eine direkte Wirkung der Aufladung auf Kationen zurückzuführen ist oder dadurch zustande kommt, dass sich die Aufladungswirkung auf dem Wege über das vegetative Nervensystem auf die Kationen erstreckt. In dieser Hinsicht haben wir die Aufladungsversuche

TABELLE III

Verhalten einiger Mineralstoffe im Serum bei der elektrischen Aufladung des Serums (Versuche in vitro)

Datum und Name		Vor Aufladung	+450 V (5 Min.)	Differenz	−450 V (5 Min.)	Differenz
19.6.52	Na (mg%)	328	325	− 3	337	+ 9
M. Fujii	K (mg%)	16.1	16.5	+0.4	15.8	−0.3
♂ 30 J.	Ca (mg%)	11.2	11.2	± 0	11.6	+0.4
24.6.52	Na (mg%)	337	335	− 2	340	+ 3
K. Kishimoto	K (mg%)	16.7	17.2	+0.5	16.4	−0.3
♀ 25 J.	Ca (mg%)	11.2	10.8	−0.4	11.2	± 0
24.6.52	Na (mg%)	356	351	− 5	368	+ 12
R. Igaki	K (mg%)	15.9	16.6	+0.7	15.7	−0.2
♀ 24 J.	Ca (mg%)	10.8	10.8	± 0	10.8	± 0
20.9.52	Na (mg%)	373	373	± 0	374	+ 1
K. Fujii	K (mg%)	19.5	19.8	+0.3	19.5	± 0
♂ 26 J.	Ca (mg%)	11.2	11.2	± 0	11.6	+0.4
29.9.52	Na (mg%)	376	370	− 6	376	± 0
M. Fujii	K (mg%)	—	—	—	—	—
♂ 30 J.	Ca (mg%)	10.8	10.4	−0.4	10.8	± 0
6.10.52	Na (mg%)	382	366	− 16	384	+ 2
S. Takeuchi	K (mg%)	24.2	24.2	± 0	23.7	−0.5
♀ 34 J.	Ca (mg%)	10.8	10.8	± 0	11.2	+0.4
10.10.52	Na (mg%)	330	330	± 0	329	− 1
H. Hisatani	K (mg%)	12.5	13.2	+0.7	12.2	−0.3
♀ 23 J.	Ca (mg%)	10.0	9.6	−0.4	10.0	± 0
	Na (mg%)	355	350	− 5	358	+ 3
Durchschnitt	K (mg%)	17.5	17.9	+0.4	17.2	−0.3
	Ca (mg%)	10.9	10.7	−0.2	11.0	+0.1

in vitro gemacht, und an normales Serum die Spannung (+450 V oder −450 V, 5 Minuten lang) angelegt und die Werte von Kationen vor und nach der Aufladung bestimmt. Die Resultate sind in Tabelle III wiedergegeben.

Wie aus Tabelle III ersichtlich ist, verschieben sich die Kationen bei der elektrischen Aufladung des Blutserums, wenn auch nicht mehr so auffallend wie bei der Aufladung des Menschen, so doch, ähnlich wie am lebenden Menschen, fast immer regelmässig nach bestimmter Richtung hin. Das Resultat in vitro lässt ohne weiteres darauf schliessen, dass auch die gesetzmässige Verschiebung der Kationen während der Aufladung, analog wie die Flockungszahl-Veränderung, hauptsächlich durch eine direkte Wirkung der Aufladung auf einzelne Mineralkomponenten zustande kommt. Ob diese Kationen-Verschiebung ursächlich etwa mit den Proteinfraktionen des Serums, die auch bei der Aufladung ebenfalls einer gewissen Verschiebung unspezifischer Art unterworfen sind, zu tun hat, schien jedoch unwahrscheinlich zu sein, da bei elektrischer Aufladung, gleichgültig, ob es sich um eine positive oder um eine negative Aufladung handelt, die Albumine immer erheblich abnehmen und die γ-Globuline dagegen stets deutlich zunehmen, während aber α- und β-Globuline keine eindeutige Verschiebungen zeigen.

Wie häufig die gesetzmässigen Verschiebungen einzelner Kationen bei der Aufladung sowohl in vivo als auch in vitro stattfinden, kann man aus folgenden Uebersichtstabellen (Tabellen IV und V) leicht entnehmen. Die gesetzmässige Verschiebung von Natrium und Kalium im Serum bei der Aufladung des Menschen erfolgt in 100% und diejenige des Calcium aber in nur etwas geringerem Ausmasse als die des Natrium und des Kalium. Das Gesetz der Aufladung gilt demnach auch für die Kationen im Blutserum.

Was die Grösse der Verschiebungen von Natrium, Kalium und

TABELLE IV

Verhalten einiger Mineralstoffe im Serum bei der elektrischen Aufladung des Menschen (Uebersichtstabelle)

		+450 Volt			−450 Volt		
		Zunahme	Abnahme	unverändert	Zunahme	Abnahme	unverändert
Na	Fälle	0	19	0	4	0	0
	%	0	**100**	0	**100**	0	0
K	Fälle	19	0	0	0	5	0
	%	**100**	0	0	0	**100**	0
Ca	Fälle	0	18	1	4	0	1
	%	0	**94.7**	5.3	**80.0**	0	20.0

TABELLE V

Verhalten einiger Mineralstoffe im Serum bei der elektrischen Aufladung des Blutserums (Uebersichtstabelle)

		+450 Volt			−450 Volt		
		Zunahme	Abnahme	unverändert	Zunahme	Abnahme	unverändert
Na	Fälle	0	5	2	4	0	3
	%	0	**71.4**	18.6	**57.1**	0	42.9
K	Fälle	5	0	1	0	5	1
	%	**83.3**	0	16.7	0	**83.3**	16.7
Ca	Fälle	0	3	4	3	0	4
	%	0	42.9	**57.1**	42.9	0	**57.1**

Calcium im Serum anbetrifft, so nehmen die Kationen bei der positiven bzw. negativen Aufladung durchschnittlich um 4–5% gegenüber den Ausgangswerten zu oder ab. Die Werte sind an und für sich nicht sehr auffallend gross. In Anbetracht der regelmässigen Verschiebungen einzelner Kationen ohne Ausnahme kann man aber mit Recht die Messungsfehler ausschliessen.

Zusammenfassung

Fassen wir unsere Versuchsergebnisse kurz zusammen, so ergibt sich

1. Bei der „positiven" Aufladung des Menschen zeigen Natrium und Calcium im Blutserum eine Verminderung, Kalium dagegen eine Vermehrung. Bei der „negativen" Aufladung zeitigen Natrium und Calcium eine Zunahme, Kalium jedoch eine Abnahme. In dieser Hinsicht zeigen die antagonistisch wirksamen Elemente auch bei der elektrischen Aufladung gerade das umgekehrte Verhältnis.

2. Auch bei der „positiven" Aufladung des Blutserums in vitro zeigen Natrium und Calcium in meisten Fällen eine Verminderung, Kalium hingegen eine Vermehrung. Bei der „negativen" Aufladung des Blutserums ist im Gegensatz zur Abnahme des Kalium eine wenn auch nicht sehr häufige Zunahme von Natrium und Calcium zu beobachten.

3. Der Aufladungseffekt ist bei der Aufladung der Versuchspersonen sehr signifikant und in allen Fällen ausnahmslos immer reproduzierbar.

4. Dieser Erfolg tritt aber nur solange auf, als elektrisch aufgeladen ist.

Es lässt sich daher mit Recht sagen, dass drei Elemente im Blutserum, d.h. Natrium, Kalium und Calcium, bei der elektrischen Aufladung mehr oder weniger deutlich nach einer bestimmten „Regel" verschoben sind.

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Responsiveness of Sweat Glands to Adrenaline in Skin Diseases. I

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The progress in the physiology of sweating has been remarkable in recent years, but from the dermatological point of view, there are as yet many problems awaiting solution. In 1948 Prof. Wada[1)2)] with his co-worker of Tohoku University, devised a method for visualizing the response of human sweat glands, by which he disproved the common notion that human sweat glands are unresponsive to adrenaline. By determining the minimal effective threshold concentration of adrenaline applied intradermally, he attempted to measure the excitability of sweat glands. In the present investigation, determination of the threshold values was performed using his method, in 971 subjects (835 patients with skin diseases, 57 patients with urogenital diseases and 79 healthy persons).

Tested subjects. Patients of our Clinic treated since the year of 1950, leprosy patients in Tohoku Shinseien Leprosorium, students and nurses.

Method of experiment. The extensor side of the forearm was painted with 2.4% iodine-absolute alcohol solution and dried completely. Then 0.2 cc. of adrenaline in various concentrations (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} in 0.9% saline solution) was injected intradermally into the treated skin. The part was then coated with a mixture of 100 g. of fine starch powder and 100 cc. of castor oil, so that any trace of sweating would show as black spots. The measurement was undertaken 15 minutes later, or in cases when no sweating appeared at time, 30 minutes after the injection. The minimal effective concentration of adrenaline when the sweating began to show in spots distributed evenly on the original wheal of injection was determined and the reciprocal of the value was taken to express the threshold. For control 0.9% physiological saline was used. The tests were made with the subject seated on a chair in most cases. The room temperature stood at 8°C–31°C. As the atmospheric humidity was sure to affect the sweating, a hygrometer was used to measure and register the humidity for taking it into consideration.

Results of experiment. See Table I.

TABLE I

Adrenaline Sweating Threshold Values in Various Dermatoses

Diagnosis	Cases	Concentrations of adrenaline in 0.9% saline solution								Percentage by groups		
		10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	Negative*	10^{-10} ~10^{-8}	10^{-7} ~10^{-6}	10^{-5} neg.
Allergic diseases												
Contact dermatitis	30			8	8	11	5	2	2	6.4	63.3	30
Eczema acutum	20			6	10	2		1	1	30.0	60.0	10
Eczema chronicum	32			3	7	14	5		3	9.4	65.6	25
Lichen Vidal	12				2	5	1	2	2		58.3	41
Neurodermitis	46	3		3	7	19	6	3	5	13.0	57.5	30
Atopic dermatitis	16	1		2	7	3	2			18.7	62.5	18
Prurigo Hebra	6			1	1	2	2			16.7	50.0	33
Prurigo nodularis	2				1	1					100.0	
Pruritus cutaneus	20	1		4	10	4	1			25.0	70.0	5
Urticaria	16			3	5	6	1	1		18.7	62.5	18
Erythema exsudativum multiforme	15	1		3	6	5				26.7	73.3	
Erythematodes	16			3	5	7			1	18.8	75.0	6
Erythema annulare	7			1		2	3	1		14.3	28.6	57
Erythema nodosum	4					2		1	1		50.0	50
Purpura	10	1		1	3	3	1	1		20.0	60.0	20
Toxicodermie	12			2	2	4	1	2	1	16.7	50.0	33
Keratodermia tylodes palmaris	5		1	2	1	1				60.0	40.0	
Weber-Christian's disease	1						1					
Seborrheic diseases												
Eczema seborrhoicum	26	1	1	6	7	7	2		2	26.8	59.2	14
Acne rosacea	9			3	5		1			33.3	55.6	11
Acne vulgaris	19			3	11	4		1		15.8	79.0	5
Hyperidrosic diseases												
Osmidrosis axillae	18			2	3	9	4			11.1	66.7	22
Hyperidrosis localis	11			2	4	3	2			18.1	63.6	18
Keratotic diseases												
Ichthyosis	15	1		4	1	4		1	4	33.4	33.3	33
Lichen pillaris	2			1	1					50.0	50.0	
Keratosis palmoplantaris hereditaria	4			1	2	1				25.0	75.0	
Porokeratosis	1											
Pityriasis rubra pilaris	2							2				100
Psoriasis	17			5	3	3	2	2		27.4	47.0	23
Erythrodermia	8			1		4	2		1	12.5	50.0	37
Bullous diseases												
Herpes zoster	10				1	4	4	1			50.0	50
Epidermolysis bullosa hereditaria	2						2					100
Dermatitis herpetiformis	3					1	1	1			33.3	66
Pigmentary diseases												
Vitiligo	20				11	3	6				70.0	30
Leucodermie post-lésionnelle	5				1	3	1				80.0	20
Nevus systematicus depigmentosus	5				2	3					100.0	
Nevus pigmentosus	22			2	7	3	7			19.1	45.4	45
Nevus fuscocaeruleus ophthalmomaxillaris Ôta	7			1	2	1	1	1	1	14.2	42.9	42
Epheliden	4			1	2	1				25.0	75.0	

TABLE I—Concluded

Diagnosis	Cases	Concentration of adrenaline in 0.9% saline solution								Percentage by groups		
		10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	Negative*	10^{-10} ~10^{-8}	10^{-7} ~10^{-6}	10^{-5}~ negative
Chloasma	4	1		1		2				50.0		50.0
Riehl's melanosis	6	1		1	1	2		1		33.3	50.0	16.7
Xeroderma pigmentosum	2					1	1					
Acanthosis nigricans	1						1					
Depilated diseases												
Alopecia areata	45	3		9	17	11	4		1	26.7	62.2	11.1
Other alopecia	6			3	1	2				50.0	50.0	
Perniosis												
Type of erythema exsudativum	4			2	1	1				50.0	50.0	
Type of cyanosis-swelling	8			1	4	3				12.5	87·5	
Acrocyanosis	3					1		2			33.3	66.7
Congenital diseases												
Hemangioma	23		1	5	7	8	1		1	26.1	65.2	8.7
Nevus anaemicus	2					2						
Cutis marmorata pigmentosa	1					1						
Multiple follicularcyste Bosselini	1						1					
Recklinghausen's disease	2				1	1						
Leprosy												
Lepra maculosa	13			1		4	5	1	2	7.7	30.8	61.5
Lepra nervosa	12				1	1	1	1	8		16.7	83.3
Lepra tuberosa	37					5	4	5	23		13.5	86.5
Lepra mixta	9				1		2	1	5		11.1	88.9
Tuberculous diseases												
Tuberculosis cutis	45	1		5	19	14	4	1	1	13.3	73.3	13.4
Tuberculosis renalis	20				5	11	2	1	1		80.0	20.0
Epididymitis tuberculosa	12	2		3	4	1	1	1		41.7	41.7	16.6
Infectious diseases												
Folliculitis superficialis	17		1	4	9	1		1	1	27.1	58.8	14.1
Furuncle	9			1	1	3	2	2		11.1	44.4	44.5
Erysipelas	3				1	1			1		66.7	33.3
Pyodermia vegetans	1								1			
Pyaemid	1								1			
Trichophytosis	21	1		4	7	5	3		1	23.8	57.1	9.1
Syphylis II latens	13			2	7	3		1		15.4	76.9	7.7
Verrucae	3			1	2					33.3	66.7	
Skin tumors												
Malignant tumor	7				4	1	1	1			70.0	30.0
Benign tumor	5			2	3					40.0	60.0	
Other diseases												
Sclerodermia diffusa	3				1	1		1			67.7	33.3
Sclerodermia en bandes	2				1	1					100.0	
Myxoedema circumscripta tuberosa	1					1						
Pellagra	3				1	1	1				66.7	33.3
Pityriasis rosea Gibert	5			1	2	2				20.0	80.0	
Skin diseases	835	20	3	115	243	235	103	41	75	16.5	57.3	26.2
Urogenital diseases	57	3		5	17	18	7	6	1	14.0	61.4	24.6
Healthy subjects	79	2	1	18	37	17	2	1	1	26.6	68.4	5.0

*, no response to 10^{-4} concentration.

Comment

Wada *et al.*[1)2)] reported that the value of excitability for any individual is constant throughout the year, standing at 10^{-7} for adults in normal health and tending to show the higher threshold at younger and older ages, although the mechanism of local sweating by adrenaline and its physiological significance are not yet clear. Sonnenschein[3)] also reported that local sweating may be caused by adrenaline and this sweating is different from sweating by acethylcholine in following three points, the latter i) accompanies activity of the axon reflex, ii) is inhibited by atropine iii) but not by dibenamine. Haimovici[4)] proposed the theory of adrenergic innervation of sweat glands but Sonnenschein[5)] and others rejected the proposition. They contended that the sweat gland cells respond directly to adrenaline and that though adrenaline and acetylcholine are chemically different, it may be that the same gland cells respond to both synergistically, rather than that different cells show respective sensitivity to the two agents. There have been found few compelling reasons to support the theory of adrenergic innervation although the theory of sympathetic innervation is prevalent at present. For the mechanism of sweating caused by intradermal injection of adrenaline, I would prefer the supposition that the gland cells respond directly to adrenaline, without the interference of the sudorific nerves, as Wada *et al.* have maintained.

The healthy control subjects in my experiment showed the threshold value of 10^{-7} in most cases, in agreement with the results by Wada[2)], Kimura[6)], etc. In various skin diseases, including every age classes from 8 to 70 years and considering the difference in humidity at the time of tests, the values of 10^{-7} and 10^{-6} were considered as normal, the values of 10^{-8}, 10^{-9}, and 10^{-10} as hyperexcitable, the values of 10^{-5} and 10^{-4} as hypoexcitable and non-response to the concentration of 10^{-4} as negative. The total outcome of the test for the adrenaline sweating threshold (hereinafter designated as A. S. T.) of all the 835 subjects with skin diseases is shown in Table I. The majority had the A. S. T. value of 10^{-7}, but the skin disease patients tend to show the higher value in comparison with the healthy controls. The same tendency was also observable in the patients with urogenital diseases. Details in various diseases are as follows:

1) Allergic diseases. In contact dermatitis patients who showed conspicuous redness and swelling as well as edema in many cases, their A. S. T. generally measured 10^{-6}, with more hypoexcitable but less hyperexcitable cases, while among the patients of eczema acutum with sporadic papules, hyperexcitable cases were somewhat more common than in the above. Hypoexcitation was remarkable among lichenous patients such as with eczema chronicum, lichen Vidal, neurodermitis disseminata and

atopic dermatitis, especially in the second and the third of the above. This fact may be accepted as natural when we remember that Kochs[7] has pointed out the disturbance of sweating secretion influenced by the vegetative nervous disharmony among atopic dermatitis patients. In pruritus cutaneus, on the other hand, the A. S. T. showed results not much different from those obtained with the healthy controls, and it may be noted that this disease with strong itching, but no eruption, showed different A. S. T. from the eruptive diseases. While among urticaria patients rather many hypoexcitable cases were found, there were few of them among patients of erythema exsudativum multiforme and erythematodes, only one case of negative response being observed in erythematodes acutus. But among erythema annulare and erythema nodosum more hypoexcitable cases were noted, while one out of two erythema annulare centrifugum Darier showed 10^{-8} and the other 10^{-5}. Thus in the patients with various exsudative erythemas a difference in excitability was observed in accordance with the difference in the type of the clinical manifestations. While in the purpura there are found equal cases of high and low A. S. T., the one case of erythema annularis teleangiectodes Majocchi had the A. S. T. of 10^{-10} and that of Schamberg's disease 10^{-6}. By toxicodermia, there were many high A. S. T. cases, but many causative factors make it difficult to discuss these results in same category. There was not a case of A. S. T. above 10^{-5} among the keratodermia tylodes palmaris progressiva patients, all being in the hyperexcitable category, and it may be recalled with interest from the view point of pathogenetic mechanism that this disease is localized on the palms where sweating is most pronounced and that Kitamura[8] has reported on the disturbance of sweating prevalent among the patients of the disease. In summary, it may be noted that the A. S. T. value in allergic patients show different tendencies according to the type of clinical manifestation of the disease.

2) Seborrheic Diseases. Eczema seborrhoicum in adult and acne rosacea patients showed hyperexcitability in most cases. These results coincide with the prevalence of primary sympatheticotony of type S. by Abe[9] of our Clinic by means of adrenaline test with blood pressure as index. By acne vulgaris, however, little difference from normal health could be found.

3) Hyperidrosic Diseases. Among the osmidrosis axillae and hyperidrosis localis patients hypoexcitable cases prevailed, giving suggestion on the pathogeny of such hyperidrosic diseases.

4) Keratotic diseases. In ichthyosis vulgaris, no difinite tendency of deviation was observable, but in keratosis palmoplantaris hereditaria no case had higher A. S. T. than 10^{-5}. This fact does not surprise us when we recall that Prof. Itô[10] of our Clinic has pointed out the clearly observa-

ble physiogenetic difference between the two diseases. Two cases of pityriasis rubra pilaris showed a conspicuous rise of A. S. T. Therefore, it is interesting that good results were obtained by Katabira[11] *et al.* of our Clinic in treating it with benzylimidazolin. Psoriasis showed both hyper- and hypoexcitability. In erythrodermia were many cases of high A. S. T. observed.

5) Bullous diseases. The epidermolysis bullosa hereditaria and dermatitis herpetiformis Duhring that are associated with disorder in pituitary adrenal system, and herpes zoster cases showed no hyperexcitability, all showing conspicuous tendency of hypoexcitability.

6) Pigmentary diseases. Most of vitiligo and leucodermie post-lésionnelle showed no hyperexcitability and all cases of nevus systematicus depigmentosus showed normal value. In nevus pigmentosus and nevus fuscocaeruleus ophthalmomaxillaris Ôta, the prevailing tendency was for hypoexcitability. No case in the chloasma showed normal A. S. T.

7) Depilated diseases. Most of the alopecia areata cases had A. S. T. of 10^{-7}, mingled with a comparatively large number of low A. S. T. cases. In other cases of alopecia the situation was similar, in parallel with the results of adrenaline test with blood pressure index by Abe[9] of our Clinic and with Tsukada's[12] observation of a tendency to vegetative nervous dysharmony in alopecia areata.

8) Perniosis. Upon classifying the cases into those of the type of erythema exsudativum multiforme and those of the type of cyanosis-swelling, the former included more numerous cases of hyperexcitability than the latter. It may be recalled that Fujisawa[14] of our Clinic reported that the former showed nearly normal capillary resistence values and the latter high values. The three cases of acrocyanosis showed hypoexcitability without exception, showing a difference existing between this disease and common perniosis.

9) Congenital diseases. The great majority of the cases had the A. S. T. of 10^{-6}, and little difference in the sweating excitability was observed by the patients from that of the normal.

10) Leprosy. As most of the cases complained of sensory anesthesia, I had to select non-anesthetic spots in many cases outside the extensor side of the forearm for the test. Many of the cases failed to respond at the dilution of 10^{-4}, showing an extreme degree of hypoexcitability. The more advanced the clinical manifestation in leprosy, the more numerous became the cases of negative response. Thus it was easy to understand that in not only the anesthetic areas but also in the non-anesthetic areas of the skin there can be found degenerated sweating function.

11) Tuberculous diseases. Kimura and Otsuji[15] reported that tuberculosis pulmonalis cases showed a tendency to higher A. S. T. in

comparison with healthy persons, a tendency especially remarkable in exudative type cases, while the cases with productive symptoms showed rather a contrary tendency to hyperexcitability. By the tuberculosis cutis patients, there was no such tendency as in tuberculosis pulmonalis cases, and little difference was found among the various disease types. However, the control patients of tuberculosis renalis showed A. S. T. of 10^{-6} in the majority of the cases, not one showing hyperexcitability, while many of the epididymitis tuberculosa patients were found to be hyperexcitable.

12) Infectious diseases. Among the folliculitis superficialis, trichophytiae, verrucae and syphilis II latens patients the A. S. T. of 10^{-7} was prevalent, and the situation was little different from that among the healthy. However, among furuncle, erysipelas and pyodermia vegetans, pyaemid cases, the more advanced the clinical manifestations, the more numerous hypoexcitable cases were found.

13) In malignant skin tumor patients comparatively more cases of hypoexcitability were observed than in benign tumors.

14) No hyperexcitable case was found among sclerodermia, myxoedema circumscripta tuberosa and pellagra patients. Not one case of hypoexcitability among pityriasis rosea Gibert cases was found.

Conclusion

In 835 cases of dermatoses, the examinations of excitability of sweat glands were performed by means of adrenaline injection and following results were obtained.

1. In allergic diseases, the excitability showed different tendencies according to the skin manifestations.
2. In seborrheic diseases and depilated diseases the excitability tends to rise in coincidence with adrenaline test with blood pressure as index.
3. In bullous diseases it falls on the contrary.
4. The leprous patients showed an extreme decrease in the excitability especially in severe cases.
5. In infectious diseases the more advanced the clinical manifestation the more numerous hypoexcitable cases were found.

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Responsiveness of Sweat Glands to Adrenaline in Skin Diseases. II

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Adrenaline sweating threshold (A. S. T.) values of 269 patients with skin diseases were examined in healthy and diseased skin area.

The method for testing adrenaline sweating was described in the proceeding section in this journal.

Results of experiment are as Table I.

Comment

1) Depigmented areas. The lesions of vitiligo showed prevalence of hypoexcitability, including some cases of negative excitability. This observation is of interest in collation with the vegetative nerve function noted in connection with the pathological mechanism of this disease. Disturbance of sewat secretion in depigmented areas has been pointed out by Burgess and Warner, Leschke, Ullmann, Leloir, Kaposi and McChabrier, while Takigawa[1] has reported anomalous results in his adrenaline wheal tests on vitiligo. The tendency to decreased sweating excitability may be accepted as natural when we remember that Prof. Itô[2] of our Clinic has observed dilation of blood vessels in his histological study on such lesions. However, the areas affected by nevus systematicus depigmentosus never showed negative nor 10^{-4} and there was no difference in the excitability of the diseased and healthy parts in most cases. From these it can be admitted that there exists appreciable difference between these two diseases in sweating excitability. Recently Halter[3] reported that the two diseases can be differenciated by the change of sweat secretion in response to heat; this is in good harmony with my observation. The cause of such a difference may be sought in the fact that whereas the vitiligo is post-natal and progressive, consequently involving various dermal functions, the nevus systematicus depigmentosus is in almost all cases limited in a certain area and has only to do with the function of melanin production without appreciably interfering with the sweating function.

TABLE I

Adrenaline Sweating Threshold Values in Various Dermatological Lesions

Lesion	Cases	Concentration of adrenaline in 0.9% saline solution							Comparison of A.S.T. values in the affected and normal skin areas			
		10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	Negative§	N†>A*	N=A	N<A	Both negative
Depigmented areas													
Vitiligo	26				6	5	6	5	4	16	9	1	
Nevus systemat. depigmentosus	7				1	4	2			2	5		
Hyperpigmented areas													
Nevus pigmentosus	16					1	3	6	6	12	3		1
Nevus systemat. pigmentosus	10			1	2	5	2			5	2	3	
Nevus fuscocaeruleus ophthalmo-maxillaris Ôta	8				1		1	4	2	8			
Xeroderma pigmentosum	2				1		1				2		
Fixed drugexanthem	1				1						1		
Depilated Areas													
Alopecia areata	39				2	8	5	12	12	34	4		1
Sensory anesthetic areas													
Lepra nervosa	24					1	1	1	21	10	2		12
Lepra maculosa	8					1	3	1	3	4	3		1
Areas with vascular disturbances													
Hemangioma	9				2	4	1	1	1	5	4		
Klippel-Weber's disease	4				1	1	2			1	3		
Nevus anaemicus	2					2					2		
Erythema exsudat. multiforme	13	1			4	2	3	2	1	3	9		1
Purpura	3	1				2					3		
Perniosis													
Type of erythema exsudativum	3	1	1			1				1	2		
Type of cyanosis-swelling	3							1	2	3			
Acrocyanosis	1		1								1		
Keratotic areas													
Ichthyosis vulgaris	8			5	1	2					5	3	
Psoriasis	10				1	2	1	2	4	8	2		
Trichophytosis	5						1	3	1	4			1
Darier's disease	1						1			1			
Keratosis follicul. squamosa Dohi	1					1					1		
Lichenificated and eczematous areas													
Neurodermitis circumscripta	4					1	1		2	4			
Neurodermitis disseminata	22				1	4	7	2	8	16	4		2
Atopic dermatitis	10				1	2	4	1	2	6	3		1
Eczema chronicum	9							3	6	8			1
Contact dermatitis	10				1	2	1	1	5	8	1		1
Eczema seborrhoicum	2					1	1			2			
Atrophied skin areas													
Sclerodermia diffusa	3				1	1			1	1	2		
Sclerodermia en bandes	2				2						1	1	
Poikilodermia	1								1	1			

†, A. S. T. value in normal skin areas, *, A. S. T. value in affected skin areas, §, No response to 10^{-4} in both areas.

2) Hyperpigmented areas. In nevus pigmentosus cases, many showed A. S. T. of 10^{-4} and negativeness, though it must be born in mind that where the nevus was deep colored, the black sweat spots in testing the diseased area were sometimes rather difficult to distinguish. In nevus systematicus pigmentosus cases, however, the lesions were generally lighter in color and the sweat spots appeared more clearly defined, and no such cases of decreased excitability was ever found. On the contrary, some cases were found to show even increased excitability in the diseased than in the healthy skin. Such obesrvations seemed to show the closer relation to the vegetative nerves of the nevus systematicus pigmentosus, sometimes called the localized nerve nevi. The nevus fuscocaelureus opthhalmo-maxillaris Ôta cases showed always decreased excitability in the diseased areas, in consonance with the results showing angioneurotic disturbance obtained by K. Yoshida[4] of our Clinic in histamine scratch tests, and with the histological observation that most prominent change of this disease lies in the corium.

3) Depilated areas. Very many cases of the areas affected by alopecia areata showed A. S. T. of 10^{-4} or negative, in most cases markedly higher than the unaffected areas. These results are deemed natural in consideration of the local disturbance of the vegetative nerve function and the histological observation of local atrophy of sweat glands by Tsukada[5] of our Clinic and the efficacy of acetylcholine in treating this disease.

4) Sensory anesthetic areas. Most of the areas of sensory anesthesia in leprosy patients showed negative response. However, in the maculae of lepra maculosa few cases of negativeness and equal A. S. T. value to the healthy skin areas were observed. These remind of the temporary hypersensitivity and paresthesia sometimes presented in lepra maculosa. Yoshimura[6] reported that the degeneration of sweating function in leprosy patients was not only due to the degenerative change of nerves, but also to the mechanical disturbances exerted by the leprous infiltration in the sweat glands and their surroundings. My findings are in harmony with such a proposition.

5) Areas with vascular disturbances. 5 cases of hemangioma showed higher A. S. T. than, and 4 cases equal to the healthy areas. One case of Klippel-Weber's disease showed higher A. S. T. than, and 3 cases equal to, the unaffected areas, but comparing the healthy part in affected extremities with the one in unaffected site, the decreased excitability in the former was shown suggestive of the disturbance of trophic nerves. All the nevus anaemicus and most of the erythema exsudativum and purpura cases showed the equal A. S. T. values in the diseased to the healthy areas.

Among the pernio cases, the erythema exsudativum multiforme type showed normal A. S. T. in two cases and hypoexcitability in one case in

the affected sites, while the cyanosis-swelling type showed 10^{-4} or negative A. S. T. in all four cases. This difference in reaction by type is in consonance with Arai's[7] report that at the skin temperature lower than 20°C when the peripheral blood vessels are paralized, no sweating could be induced by adrenaline administration.

6) Keratotic areas. Such areas in ichthyosis vulgaris mostly showed the A. S. T. of 10^{-8}, manifesting a decided hyperexcitability and in no case the A. S. T. was higher in the diseased than in the healthy parts, a phenomenon rarely duplicated in other skin diseases. Prof. Itô[8] has already pointed out that this disease has the disturbance of sweating function as one of its important causative factors, in consideration of the facts that it tends to be alleviated in summer when sweating is most plentiful and that pilocarpine injection showed therapeutic effects in some cases. My results above also endorse the Itô's observations about the disturbance in sweating function. In psoriasis cases, the A. S. T. value in the diseased parts showed a tendency to rise, in most cases to the extent of 10^{-4} and negativeness. Ichthyosis vulgaris and psoriasis are both known to prefer to attack the extensor surfaces of the extremities and it is worthy of special note that these two diseases are opposite in their response to adrenaline.

7) Lichenificated and eczematous areas. In neurodermitis circumscripta and disseminata, eczema chronicum, contact dermatitis and eczema seborrhoicum cases, the A. S. T. tended to rise in the diseased areas. We are reminded of the reports by Sulzberger[9], Louis Tuft[10] *et al.* to the effect that, considering the stagnation of sweating due to the horny plugging of the opening of sweating pores in the diseased areas, disturbance of sweating is an important pathogenic factor of atopic dermatitis.

8) Atrophied skin areas. Two out of three cases of sclerodermia diffusa showed equal A. S. T. value to unaffected and the one case higher value than healthy areas, but sclerodermia en bandes showed no higher value in involved lesions. The one poikilodermia case showed higher A. S. T. value in their diseased areas.

Conclusion

The results of A. S. T. test in the affected areas in various dermatoses are as follows:

1. Depigmentation, Hyperpigmentation, alopecia, sensory anesthesia, lichenification, eczema and atrophy cases had decided hypoexcitability than in the healthy skin areas of the same subject.

2. Erythema exsudativum and purpura cases showed almost no difference in A. S. T. in both diseased and healthy skin areas.

3. The pernio cases especially cyanosis-swelling type showed hypoexcitability.

4. Comparing the healthy part in affected extremities with that in unaffected site in Klippel-Weber's Disease patients, the decreased excitability in the former was shown suggestive of the disturbance of trophic nerves.

5. Ichthyosis vulgaris showed strong hyperexcitability in the diseased areas, moreover some cases presented lower A. S. T. values than in the healthy areas.

6. Psoriasis cases, on the contrary, showed a marked hypoexcitability.

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Responsiveness of Sweat Glands to Adrenaline in Skin Diseases. III

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I. Change of A.S.T. accompanying the Progress of the Malady (See Table I)

Wada-Takagaki's A.S.T. (adrenaline sweating threshold) tests were conducted on 57 skin and urogenital disease patients before and during their treatments and after recovery for the purpose of following up the change in their A.S.T. According to Wada[1], the A.S.T. values in healthy adults showed no change throughout the year, but Kimura[2] and Ôtsuji[3] admitted some seasonal variation in A.S.T. values in patients and reported that the change in A.S.T. values may be serviceable in prognostication in some cases of diseases such as tuberculosis. In my findings, normalization of A.S.T. means the approximation to the value of 10^{-6} and 10^{-7} either by rising from a lower or falling from a higher A.S.T., and 22 cases that showed such a normalization presented simultaneous improvement in their situation without exception. On the other hand, denormalization means further deviation from the normal values of 10^{-6} and 10^{-7}, either by rise or fall of A.S.T., and 3 cases showing such denormalization by rise of A.S.T. showed aggravation in their progress, but no such aggravation was observed in denormalized cases by the fall of A.S.T., cases showing no change in A.S.T. generally showed little change in their progress either. Such minor surgical operations as extirpation of affected site in Osmidrosis axillae, epididymectomy and implantation of cow's pituitary gland did not affect the A.S.T., but one tuberculosis renalis patient retained a high A.S.T. even three weeks after nephrectomy, without coming down to the normal value. The alopecia areata multiplex patients who had improved in their situation by implantation of cow's pituitary gland usually showed normal A.S.T. values in affected areas and negative in Thorn's tests. From these facts it is suggested that the function of vegetative nerves was adjusted by that treatment.

TABLE I

Change of A.S.T. Value accompanying the Progress of the Malady

Diagnosis	Total cases	Normalization	Denormalization	No change
Eczema chronicum	3			3
Neurodermitis disseminata	2	2		
Erythema exsudat. multiforme	2	1	1*	
Erythema annulare centrifugum	1			3
Erythematodes	3	1	2*	
Toxicodermie	1	1		
Prurigo nodularis	1	1		
Purpura rheumatica	1		1	
Erythrodermia desquamativa	2	1	1	
Urticaria acuta	1	1		
Eczema seborrhoicum	1	1		
Acne rosacea	1	1		
Psoriasis	2	2		
Parapsoriasis guttata	1	1		
Tuberculosis cutis	9	1	2	6
Vitiligo	1	1		
Alopecia areata	9	2	4	3
Pityriasis rubra pilaris	1		1	
Fungus poisoning	1		1	
Mycosis fungoides	1		1	
Syphilis II latens	1		1	
Diabetes insipidus	1	1		
Osmidrosis axillae	2			2
Epididymitis tuberculosa	5	3	2	
Tuberculosis renalis	3		1	2

Gothic type: Recovery or improvement. *: Aggravation. Other cases: No improvement.

II. Variation of A.S.T. by Sites of the Body

The difference, if any, in the A.S.T. values of skin areas in the extensor surface of the forearm and the bend of elbow, the extensor and flexor surfaces of the forearm, the flexor surface of the leg and the popliteal space in undiseased conditions were measured by 121 skin disease patients and 30 healthy controls (Table II). Kuno[4] had reported that the quantity of sweating was different according to the bodily positions, but Arai[5] reported that the excitability of sweat glands was individually constant in any part of the skin, except the face and the backs of the hands and

feet, in his tests with adrenaline and acetylcholine. In my tests on 30 subjects in normal health, the results showed no difference in the A.S.T. on the extensor side of the forearm and the bend of elbow, and in the extensor and flexor surfaces of the forearm. Similarly, the tests on 6 healthy subjects revealed that the A.S.T. values on the flexor side of the leg and the popliteal space were of the same. No difference of A.S.T. value by sites was observed among about 30 kinds of dermatoses patients (78 cases) but some dermatoses that presented different values by sites were shown in Table II. Namely, 28 of 39 tested cases showed difference, and it is noteworthy that in many cases of neurodermitis, atopic dermatitis and eczema seborrhoicum the A.S.T. at the bend of elbow, the predisposing area of these diseases, was found to be higher value than that on the extensor side of the forearm. 14 out of the 29 tested cases showed no differ-

TABLE II

Differences of Sweat Glands Excitability to Adrenaline by Sites

Diagnosis	Comparison of A.S.T. values							
	Cases	a*<b†	a>b	a=b	Cases	b<c‡	b>c	b=c
Neurodermitis	10	7	1	2	7	3		4
Atopic dermatitis	3	1						
Eczema seborrhoicum	9	7	1	1	10	3	1	6
Prurigo nodularis	1	1		0	1	1		0
Eczema chronicum	3	2		1				
Ichthyosis vulgaris	3	1		2	3	1		2
Erythematodes	3	2		1	4	1	2	1
Perniosis	1	1		0	2	1		1
Keratosis palmoplant. hered.	1	1		0				
Pityriasis rubra pilaris	1		1	0				
Erythema scalatinif. recid.					1		1	0
Acne rosacea	4	2		2				
Acne vulgaris					1		1	0
	39	25	3	11	29	10	5	14

*, A.S.T. value in bend of elbow, †, the value in extensor surface of forearm, ‡, the value in flexor surface of forearm, >the former shows lower A.S.T. value than the latter, <, the former shows higher value than the latter, =, both areas show equal value.

ence in the excitability in the extensor and the flexor sides of the forearm. But 15 showed some difference, the neurodermitis, eczema seborrhoicum, ichthyosis vulgaris and prurigo nodularis cases showed decreased excitability in the extensor side, the side preferred by the diseases to the

other. Only 2 out of the tested 11 cases showed any difference in the A.S.T. in the leg and the popliteal space.

III. Relation of A.S.T. to Aschner's Phenomenon

Before testing 585 subjects with skin diseases for their A.S.T., the change in their pulsation by eyeball pressure was measured, those with decrease of more than 20 pulses per minute being classed as positive and those with decrease of below 20 per minute as negative. This Aschner's test is used as an auxiliary method in diagnosing hypertony of parasympathetic nerves, of which the resultant decrease of pulse frequency is an index. As shown in Table III, no relationship between the A.S.T. and the results of Aschner's tests was found so that it became clear that the A.S.T. is no index to the degree of parasympathetic nervous tension.

TABLE III

Relation of A.S.T. Value to Aschner's Phenomenon

Aschner's p.	Cases	A. S. T.										
		Concentration of adrenaline in 0.9% saline solution								Percentage by groups		
		10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	Negative	10^{-10} ~10^{-8}	10^{-7} ~10^{-6}	10^{-5} ~negative
Positive	142	5		18	29	56	15	9	10	16.3	59.9	23.8
Negative	443	10	2	63	147	129	45	27	20	16.9	62.3	20.8

Subjects with decrease of more than 20 pulses per minute are classed as positive, and those with decreases less than 20 per minute as negative.

IV. Comparative Tests of Adrenaline and Acetylcholine for their Excitation of Sweat Glands

The tests were conducted in the same manner as described in the first report on the undiseased parts of extensor surface of forearms with 52 skin disease patients, using adrenaline in one side and acetylcholine in other side.

Kimura[6] had tested the excitablity of sweat glands of 366 healthy subjects with adrenaline and acetylcholine and found a positive relation between the values obtained with the two agents, giving the normal values of 10^{-7} for adrenaline and 10^{-13} for acetylcholine. Ôki[7] had reported that the high-aged subjects with high A.S.T. showed also high threshold of sweating with acetylcholine. Akasaka[8] has measured the time taken for incipient sweating after injection of adrenaline, acetylcholine and pilocarpine in a certain concentration in the diseased areas of skin disease patients and in control healthy parts, and found that the three formers presented parallel effects each other. In my tests, I found that the sub-

TABLE IV

Comparative Tests of Adrenaline- and Acetylcholine-Sweating Threshold Values

Concentration of adrenaline	Concentration of acetylcholine								
	10^{-19}	10^{-18}	10^{-17}	10^{-16}	10^{-15}	10^{-14}	10^{-13}	10^{-11}	*Negative
10^{-10}	1		2	5					
10^{-9}				1					
10^{-8}		1	4	5	1	8	3		
10^{-7}						1	6		
10^{-6}			1	1		2	5		
10^{-5}								1	
Negative†									4

*, No response to 10^{-10} concentration, †, No response to 10^{-4} concentration.

jects with A.S.T. of 10^{-7} or 10^{-6} showed the threshold value of 10^{-18} with acetylcholine, which I take for the normal values, and also found a parallelism between the higher and lower A.S.T. values and the higher and lower acetylcholine threshold values.

Kimura[2] and Ôtsuji[3] have assumed that the A.S.T. values obtained by the tests in the manner I have adopted express the individual reaction to adrenaline and can be used as an index of his vegetative nerve tension, but Kuno[4] has countered them in pointing out the indecisiveness of any attempt as distinguishing the nerve systems through the actions of adrenaline and acetylcholine, seeing that these two chemicals exert similar effects upon the same sweat glands. My results also showed that A.S.T. values have no definite relation with the results of Aschner's tests as mentioned above and that adrenaline and acetylcholine show similar tendency in their actions against the sweat glands. Abe's[9] adrenaline tests with the blood pressure as index also failed to establish a specific relation between their results and A.S.T. values. In summary, it is impossible to assert that the A.S.T. value commensurates with the reaction against adrenaline of the individum, or in other words, that A.S.T. can be used as an index to the degree of his vegetative nerve tension.

CONCLUSION

From what mentioned above it have been established that,

1. The skin disease patients who showed normalizing tendency in their A.S.T. showed improvement in their situation, while those who showed no variation of A.S.T. value presented no improvement in their progress.

2. The neurodermitis, the atopic dermatitis and the eczema seborrhoicum cases showed a tendency to higher A.S.T. on the bend of elbow rather than on the extensor side of the forearm, and on the extensor side rather than on the flexor side of the forearm.

3. It was inadmissible to infer the individual reaction against adrenaline from the results of these tests, judging from the points as follows (i) the A.S.T. values showed no definite relation with the results of Aschner's tests, (ii) adrenaline and acetylcholine showed similar tendency in their action on the sweat glands, (iii) and the results of adrenaline tests with the blood pressure of the subject as index stood in no apparent relation with the A.S.T. values.

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Studies on High-molecular Placental Components in Connection with Pregnancy Toxemia

First Paper*

By

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To elucidate the etiology of toxemia of late pregnancy, there have been put forward several theories hormone-, embryo-, hyperproteinemia-, dietetic deficiency-, placenta- and ishemia-theory, although none appears to have been firmly established.

Not a few authors ascribe the toxemia of late pregnancy to an allergic disorder, judging from the clinical symptoms as well as autopsy findings in eclampsia and premature separation of placenta. The opinion that placental substances absorbed causes it was expressed by Liepmann[1a] as early as 1902 and since then the placental allergen was demonstrated in the blood serum, urine and also umbilical blood of pregnant women. Regarding the placental components, Magara[2] claimed in 1942 that a polypeptide is the allergen concerned, but more recently in 1950 Kaku[3] contradicted him, insisting a polysaccharide to be the allergen. The present writers have undertaken to decide whether the polypeptide or the carbohydrate or any other or others function as the allergen. With the aim extracts of placentas once dehydrated were fractioned, and a neutral carbohydrate, glacial acetic acid-soluble and acid-precipitable proteins, a mixture of nucleic acids and an itinsulfuric acid were obtained. And the products, which were not pure enough, were examined with respect to their antigenicity and other properties capable of suggesting their relationship with pregnancy toxemia (precipitin reaction with the serum of pregnancy-toxemic women, Schultz-Dale reaction, skin reaction, effects

* Read before the 5th General Meeting of the Japanese Obstetrical and Gynecological Society, May 10–12, 1953, Niigata.[1]

on pregnant rabbits after injection particularly induction of histological lesions and else).

Experimental

Preliminary Fractionation of the Water-extractible Components of Placenta

A placenta was freed from membranes and big blood-vessels and after rough washing with water, minced. The tissue pulp was covered under about 5 volumes of 95% alcohol for about 1 week and dried in a Faust apparatus. The material obtained weighing 50 g. in averages was then refluxed with water for 1 hour. The solution was distilled in vacuo to about 1/10–1/15 the volume and $(NH_4)_2SO_4$ was added to half saturation. The deposit (**Fr. I**) was set aside after centrifugation. The supernatant which contained floating particles was filtered. The filtrate was saturated with additional $(NH_4)_2SO_4$ and a small deposit was again filtered off. The filtrate here was dialyzed for about 1 week, distilled in vacuo to a syrup and precipitated with an excess of alcohol—**Fr. II.** On the other hand, Fr. I was subjected to the procedure of Kerr & Seraidarian,[4] namely, it was taken up in 3 *M* NaCl and after rejecting the insoluble part, adjusted to pH 4.2 with dilute HCl, then the occurring precipitate (**Fr. III**) was centrifuged and the supernatant precipitated with 3 volumes of alcohol. After separating the deposit (**Fr. IV**), the mother fluid was vacuum-distilled to about 1/5 the volume to expel off most of the alcohol, dialyzed and precipitated with alcohol. The deposit here will be referred to as **Fr. V.**

Separation of the Components

Neutral carbohydrate (Ch.). Fr. II was dissolved in water and shaken repeatedly with a chloroform-amylalcohol (10 : 1 by volume) mixture to eliminate proteins according to Sevag. When no more gel formed, the centrifuged upper layer was siphoned out and an excess of alcohol was added. The deposit was wahsed with alcohol and dried in a vacuum desiccator over $CaCl_2$.

Protein I. Fr. III was taken up in the sat. NaCl and after rejecting the insoluble part, adjusted to pH 4.2 with 1% HCl. The occurring deposit was agitated in the sat. NaCl, adding drop by drop of 1% NaOH to neutral, and the insoluble removed. Again 1% HCl was added to the solution to pH 4.2 for precipitation. Redissolution and reprecipitation was further processed again and again until a deposit showing only faint Molisch reaction was obtained. It was washed with the sat. NaCl acidified to pH 4.2 with HCl, dialyzed for 5 days against running

water and vacuum-distilled to a thick solution, followed by standing in a vacuum desiccator over $CaCl_2$ to complete dryness.

Protein II. Fr. V not only showed the positive biuret (##), Molisch (##), Neuberg-Saneyoshi (+) and Goldschmiedt (+) reactions but also proved to contain phosphorus (++). It appeared thus to be a mixture of a neutral carbohydrate, nucleic acids, an itinsulfuric acid[5] and proteins. Hence it was at first washed well with the sat. $(NH_4)_2SO_4$ and the residue was dissolved in water, dialyzed and recovered by vacuum distillation and addition of alcohol. Then, the substance was dissolved again in water and the sat. $Ba(OH)_2$ was added to pH 9.0, whereby a precipitate occurred. After centrifuging off of the precipitate, the supernatant was acidified to pH 2.0 with dilute HCl, whereby again a precipitate came out which was also separated off. Moving of the pH hither to thither was repeated, and when the precipitable remained no more, the solution was freed from barium quantitatively by the aid of H_2SO_4, with following dialysis, condensation and alcohol-precipitation. The deposit dried was kneaded with a little water into a paste and an excess of 99% acetic acid was added in portions to carry down the neutral carbohydrate and itinsulfuric acid. The centrifuged supernatant was precipitated with more than 3 volumes of ether. The deposit here was further treated similar to above three more times, employing 90, 80 and 60% acetic acid in turn instead of the 99% acid. The final ether-deposit was dissolved in water and carried down with trichloroacetic acid (10%). After several reprecipitations, the substance was taken up in water and dialyzed. On vacuum-distillation of the solution some precipitate was given halfway. This was rejected and the solution was further concentrated to syrupy consistency, precipitated with alcohol and dried as above.

Nucleic acids. Fr. IV which contained an abundant quantity of nucleic acids (P ++, Dische ##, Bial ++) beside an itinsulfuric acid (Neuberg-Saneyoshi ++) and proteins (biuret ##), was dissolved in water and 5% $CuCl_2 \cdot 2H_2O$ was added until no more precipitate occurred. Centrifuged. The supernatant—**Fr. VI**—was set aside for separation of the itinsulfuric acid (S. below). The centrifugate was washed with a $CuCl_2$ solution of like concentration as the supernatant, then with HCl-containing alcohol to remove copper and finally with alcohol. After drying, it was dissolved in water (10 cc. per 100 mg. of the substance) and treated with a chloroform-amylalcohol mixture as above according to Sevag thoroughly. The solution was then dialyzed for 3 days and precipitated with alcohol containing sodium acetate, and the deposit was washed with plain alcohol and dried to a constant weight.

Itinsulfuric acid. The centrifuged supernatant of the cupric nucleates above (Fr. VI) was precipitated with alcohol, freed from copper with

HCl-containing alcohol, and after washing with alcohol, dried. The substance obtained was subjected to Sevag procedure and the alcohol-precipitate of the solution which was free from proteins was dried as above in a desiccator.

Many runs of preparation were carried out, and in averages 90, 170, 120, 30 and 50 mg. respectively of the neutral carbohydrate, Proteins I and II, nucleic acids and itinsulfuric acid were yielded from 50 g. of the placental powder. The corresponding products were united.

Physical and Chemical Properties of the Products

Qualitative test-tube tests. The results are tabulated as follows:

Test	Products				
	Ch.	Protein I	Protein II	Nucleic acids	Itinsulfuric acid
Biuret	±	##	##	−	−
Molisch	++	±	+	+	++
Goldschmiedt	±	−	−	−	++
Neuberg-Saneyoshi (for hexuronic acid)	±	−	−	−	++
P	±	−	−	##	−
Bial (for pentose)	+			++	++
Dische (for dexoxy-pentose)	+			++	
Hydrolysable S					+
Purine (ammoniacal silver test)	±			++	
Osaki-Turumi					
Indirect	±				+
Direct	−				−

− : negative, ± : faintly positive, + : slightly positive, ++ : markedly positive, ## : strongly positive.

Electrophoresis. Electrophoretical examination was made in a Tiselius apparatus. The patterns given (Fig. 1) showed that Protein II was a mixture of nearly equal amounts of two proteins and the other products contained more or less of a contamination or contaminations.

Paper partition chromatography of the sugar components of the neutral carbohydrate (a polypeptide-polysaccharide complex). Masamune & Yosizawa's procedure[6] was adapted. The carbohydrate taken for hydrolysis was 35 mg. The hydrolysate by 1 *N* H_2SO_4 was freed from most of the mineral acid and concentrated to about 0.3 cc., and 0.035 cc. was sampled. The chromatogram given is reproduced diagrammatically in Fig. 2, which shows that the material contained chondrosamine, glucosamine, galactose, glucose, mannose and a pentose.

Fig. 1

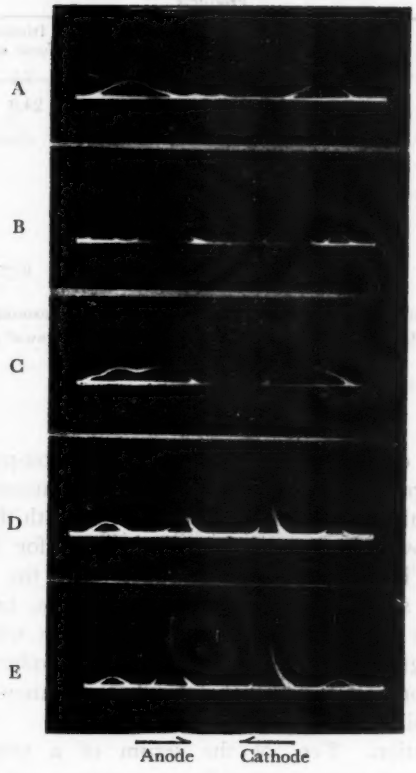

Fig. 2

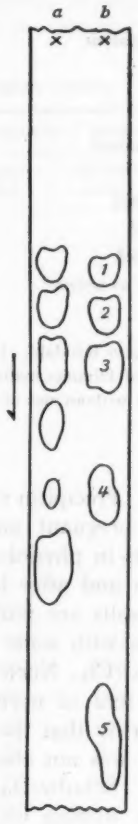

Fig. 1. Electrophoretic patterns of 1% solutions of Neutral carbohydrate (A), Protein I (B), Protein II (C), Nucleic acids (D) and Itinsulfuric acid (E) in phosphate buffer of pH 7.8 and I 0.29. A: temperature 19°C; current 26.5 mA; exposure after starting current 55 minutes. B: temp. 20.3°C; cur. 20 mA; expos. 50 min. C: temp. 20.3°C; cur. 21 mA; expos. 50 min. D: temp. 20.3°C; cur. 21.8 mA; expos. 40 min. E: temp. 10°C; cur. 15 mA; expos. 35 min. Ascending limbs to the left.

Fig. 2. A sugar chromatogram of Neutral carbohydrate obtained by descending irrigation with butanol-pyridine-water (5 : 3 : 2) for 24 hrs. Temperature of test 25°C. Indicator aniline hydrogen phthalate of Partridge. The standard solution was 1% with respect to individual sugars and 0.025 cc. of it was sampled.

a: main run, b: reference run, 1: chondrosamine hydrochloride, 2: glucosamine hydrocholoride, 3: galactose, 4: mannose, 5: rhamnose.

Quantitative analysis. The figures (percentages) below were obtained.

Analysis	Products				
	Ch.	Protein I	Protein II	Nucleic acids	Itinsulfuric acid
N*	4.3	12.9	14.6	12.0	3.1
Hexosamine†	14.9	2.9	5.9		24.8
P‡				4.9	
Galactose§	30.4				
Glucose§	9.3				
Mannose§	6.0				
Pentose as xylose//	13.1				
Ash	3.0¶	2.0¶	7.2¶	19.2**	9.2¶

* Micro Kjeldahl, † Hamasato & Akakura, ‡ Haneda, Sinokawa & Yasuoka's modification of Plimmer method, § Masamune & Ogawa[7], // Masamune & Ogawa[8], ¶ Pregl method without use of H_2SO_4, ** Pregl.

Biological Assays

1) Precipitin reaction of the products with the sera of non-pregnant, normal-pregnant and pregnancy-toxemic women. 2% solutions of the products in physiological saline were diluted progressively with the saline solution and after laying over the sera above, stood in air for 4 hours. The results are illustrated in Table I. The products gave the positive reaction with some of the sera of pregnancy-toxemic women, but three of them (Ch., Nucleic acids and Itinsulfuric acid) reacted also with some of the sera of normal-pregnant women though less in number. It is noteworthy that the reaction with the sera of seriously pregnancy-toxemic women was not always positive.

2) Schultz-Dale reaction. 3 cc. of the serum of a pregnancy-toxemic woman were injected intraperitoneally to a guinea pig of 200–300 g. body weight. After 24–48 hours from the injection, the animal was killed by bloodletting and a part of the intestine was cut out and hung in 10 cc. of Tyrode solution of 37–38°C which was being bubbled with oxygen (Magnus apparatus). And, when the intestine showed constant peristalsis, 5–10 mg. of one of the products, dissolved in 1 cc. of Tyrode solution, were added to the bath to see if it causes any contraction of the tissue. The others were assayed also on the same tissue in a similar manner, renewing each time the fluid in which the tissue was suspended. None of the products gave a positive result.

3) Pathological changes in liver and kidney of pregnant rabbits after intravenous injection of the products. 5 or 10 mg. per kg. body weight of each of the products were dissolved in physiological saline and

TABLE IA

Precipitin Reaction with the Sera of Non-pregnant, Normal-pregnant and Pregnancy-toxemic Women of the Neutral Carbohydrate

−: no reaction, ±: faintly positive r., +: slightly positive r.

No. of individuals	Dilution of products (1:)				Symptoms of patients		
	5×10	10^2	1.5×10^2	2×10^2	Blood pressure (mm. Hg)	Albuminuria	Edema
Sera of non-pregnant women							
1	−	−	−	−			
2	−	−	−	−			
3	−	−	−	−			
4	±	−	−	−			
5	−	−	−	−			
Sera of normal-pregnant women							
6	+	−	−	−			
7	±	−	−	−			
8	−	−	−	−			
9	−	−	−	−			
10	−	−	−	−			
Sera of pregnancy-toxemic women							
11	+	±	±	−	160–110	(+)	(+)
12	−	−	−	−	200–140	(卄)	(卄)
13	±	−	−	−	182–130	(卄)	(+)
14	±	−	−	−	155– 95	(卄)	(+)
15	−	−	−	−	150– 88	(−)	(卄)
16	−	−	−	−	135– 70	(卄)	(+)
17	−	−	−	−	162–100	(+)	(−)
18	+	±	−	−	220–145	(卄)	(卄)
19	+	±	−	−	165–110	(+)	(+)

injected into the auricular or portal vein of a rabbit of late pregnancy and after 24 or 48 hours, the animal was killed. The tissues were fixed with formalin or with alcohol and stained by hematoxylin-eosin, van Gieson, Weigert (for fat staining) and Kawamura-Yazaki (for fibril staining) methods. The control was carried out on non-pregnant female rabbits, in whose tissues almost no changes occurred. The histological findings in the main experiment are embodied in Table II. In liver, necrosis occurred by any of the products. The change was caused comparatively easily by the nucleic acid and itinsulfuric acid fractions. And,

TABLE IB

Precipitin Reaction with the Same Sera as Those in Table IA of the Remaining Products

Notations signify the same as in Table IA.

No. of individuals \ Product / Dil. (1 :)*	Protein I				Protein II				Nucleic acids				Itinsulfuric acid				Symptoms of patients		
	5× 10	10^2	1.5 ×10^2	2× 10^2	5× 10	10^2	1.5 ×10^2	2× 10^2	5× 10	10^2	1.5 ×10^2	2× 10^2	5× 10	10^2	1.5 ×10^2	2× 10^2	Blood pressure (mm. Hg)	Albuminuria	Edema
Sera of non-pregnant women																			
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			
21	−	−	−	−	±	−	−	−	−	−	−	−	±	−	−	−			
22	−	−	−	−	−	−	−	−	±	−	−	−	±	−	−	−			
23	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			
24	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−			
Sera of normal-pregnant women																			
25	−	−	−	−	−	−	−	−	±	−	−	−	±	±	−	−			
26	±	−	−	−	−	−	−	−	−	−	−	−	+	±	−	−			
27	±	±	−	−	±	−	−	−	±	−	−	−	±	−	−	−			
28	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−			
29	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			
Sera of pregnancy-toxemic women																			
30	−	−	−	−	±	±	−	−	−	−	−	−	+	−	−	−	160–100	(−)	(++)
31	−	−	−	−	+	−	−	−	+	−	−	−	+	+	±	±	130– 85	(+++)	(+)
32	±	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	190–100	(++)	(++)
33	±	−	−	−	−	−	−	−	+	±	−	−	+	±	−	−	186–120	(+++)	(+)
34	+	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	140– 90	(+)	(++)

* Dilution of the preparations.

TABLE II

Pathological Lesions in Liver and Kidney of Rabbits of Late Pregnancy after Intravenous Injection of the Products

TABLE II

Pathological Lesions in Liver and Kidney of Rabbits of Late Pregnancy after Intravenous Injection of the Products

Excepting the cases particularly mentioned, the substances were injected into the auricular vein and the animals were killed after 24 hrs. from injection. − : no change, + : slight change, ++ : marked change.

Dosage (mg.) per kg. body weight	Rabbit No.	Rabbit Weight (g.)	Liver: Necrosis	Liver: Necrobiosis	Liver: Cloudy swelling	Liver: Degeneration	Liver: Stasis of blood stream	Liver: Thrombosis	Kidney: Nephrosis	Kidney: Glomerulonephrosis	Premature delivery
Product injected: Ch.											
5	1	1940	−	−	−	++	+	−	+	−	−
	2	3200	−	+	+	−	−	−	−	−	+
	3	2890	−	−	+	−	+	−	−	+	−
	4*	2700	+	+	++	+	+	+	+	−	−
10	5	2440	+	+	++	+	+	−	−	+	−
Product injected: Protein I											
5	6	2740	+	−	−	−	−	−	−	−	−
	7	2720	−	−	−	+	−	−	−	−	−
	8	2810	−	+	++	−	−	−	−	−	+
	9*	3200	++	++	++	++	−	+	+	−	−
10	10	2050	−	+	++	−	+	−	+	−	−
	11	2040	−	+	+	+	−	−	+	−	−
Product injected: Protein II											
5	12	3090	−	−	++	−	−	−	+	−	−
	13	3050	−	−	+	++	+	−	+	−	−
	14	2600	−	+	+	+	−	−	−	+	−
	15*	2560	++	+	++	++	+	+	+	−	−
10	16	2330	−	+	++	++	−	−	+	−	−
Product injected: Nucleic acids											
5	17	2850	+	−	+	−	−	−	+	++	−
	18	3180	+	−	−	+	−	−	+	−	+
	19	3150	−	−	+	++	−	−	−	−	−
	20*	2560	+	−	+	+	+	−	−	−	−
10	21	1870	−	+	+	+	+	−	+	−	−
Product injected: Itinsulfuric acid											
5	22	2160	+	++	+	−	−	−	−	−	+
	23	3050	+	−	+	+	+	−	−	−	−
	24	2900	−	+	++	−	−	−	−	−	−
	25*	2880	++	−	++	++	+	+	+	−	−
10	26	2470	−	++	+	++	−	−	+	−	−
	27	1970	−	++	++	−	−	−	+	−	−

* Substance was injected into the portal vein and animal was killed after 48 hrs.

in kidney, nephrosis or glomerulonephrosis was induced also by all the products, of which Nucleic acids, Neutral carbohydrate and Protein II appeared to be more poisonous than the others. However, none of the products gave rise to the characteristic allergic changes of Rössle.

4) Schwartzman reaction. B. coli com. was cultured in an agar medium for 24 hours. Then it was inoculated in bouillon of pH 7.3–7.4 (2 platinum loops for 10 cc.) and stood in an incubator at 37°C for 5 days, followed by straining through a Seitz filter. The filtrate was kept sterile in a refrigerator and used within 10 days. a) Examination of the preparatory potency of the products for Schwartzman reaction. 0.3 cc. portions of the B. coli com. filtrate were intracutaneously injected at symmetric two points in the epigastric region of a rabbit and 0.3 cc. portions of a 0.1% solution of one of the products in physiological saline at two symmetric points in the hypogastric region and after 24 hours, 1 cc. of the bacterial filtrate per kg. body weight was injected into the auricular vein. The result was judged after 5 hours (Table IIIA). Proteins I and II and Nucleic acids were seen to have skin-preparatory potency for the bacterial filtrate intravenously injected.

b) Examination of the reacting potency of the products in the phenomenon. The preparatory injection was processed as above and the reacting injection by replacing 1% solutions in physiological saline of the products for the bacterial filtrate. As is illustrated in Table IIIB, the areas preparatorily injected showed the positive reaction after reacting injection of Proteins I and II.

5) Skin reaction. The products were separately dissolved in 1500 volumes of physiological saline containing 0.5% phenol. 0.1 cc. portions of the solutions were injected intracutaneously to non-pregnant, normal-pregnant and pregnancy-toxemic women, and the longest and shortest diameters of the reddened injected-areas were measured after 4 hours. No noticeable discrepancy of the area was shown by any of the products among the different groups of women. A study with 0.05% solutions will be described in detail in a forthcoming paper.[9]

6) Effect of the products on blood pressure of pregnant rabbits. If a female rabbit is sensitized during pregnancy by placental specific components, her blood pressure might be lowered by injection of at least some of the products as an anaphylactic reaction. Therefore, an urethane-narcotized pregnant rabbit was fettered on her back and the intravascular pressure in the carotic artery was recorded on a kymograph, injecting 3 cc. of a solution in physiological saline containing 5 mg. per kg. body weight of one of the products into the auricular vein. But, contrary to expectation, no depression took place by any of the products (Table IV).

7) Spasm in the arterioles of retina, albuminuria and premat-

TABLE IIIA

Skin-preparatory Potency of the Products for Schwartzman Reaction

−: no change, ±: faint hemorrhage, +: slight hemor. or a petechia, ++: marked hemor. with or without necrosis of the center of swelling, #: intense infiltration and swelling of the injected area with dark red necrosis and hemor.

Rabbit			Preparatory inj.			Reacting inj.	Judgement of the assay			
			Epigast. reg.	Hypog. reg.			Epig. reg.		Hypog. reg.	
No.	Body weight (g.)	Sex	Left Right	Left Right			Left	Right	Left	Right
28	2300	♀	Injected were 0.3 cc. portions of the B. coli com. filtrate	Injected were 0.3 cc. portions of 0.1% solutions of	Ch.	Injected was 1 cc. of the bacterial filtrate	++	#	−	−
29	2800	"					±	#	−	−
30	1500	"					−	+	−	−
31	1700	"			Protein I		−	++	−	++
32	1600	"					#	#	#	#
33	2100	♂					++	++	#	#
34	1900	♀			Protein II		#	#	#	++
35	1600	"					++	++	++	−
36	2400	"					±	±	−	+
37	1600	"			Nucleic acids		+	#	−	±
38	1300	"					++	++	−	−
39	1800	"					#	#	#	#
40	2500	"			Itinsulfuric acid		+	±	−	−
41	2900	"					#	#	−	−
42	1600	"					++	#	−	−

ure delivery of the pregnant rabbits injected with the products. In pregnancy toxemia particularly eclampsia and pre-eclampsia there is seen the spasm in the central artery of retina. Hence, into the auricular vein of pregnant rabbits were injected a 5 or 10 mg. portion per kg. body weight of the placental products after dissolution in 5 cc. of physiological saline, to observe the blood vessel of retina by direct ophthalmoscopy after 30 minutes and after 24 hours from the injection. None of the products caused the spasm, whereas premature delivery occurred by all of them exclusive of Protein II as shown in Table II. Albuminuria did not happen.

SUMMARY

1. High-molecular substances (a neutral carbohydrate, a protein precipitable at pH 4.2 (Protein I), a mixture of proteins soluble in glacial acetic acid (Protein II), a mixture of nucleic acids (PNA and DNA) and

TABLE IIIB

Reacting Potency of the Products for Schwartzman Reaction

Notations signify the same as in Table IIIA.

Rabbit			Preparatory inj.			Reacting inj. (Product and amount inj.)	Judgement of the assay			
No.	Body weight (g.)	Sex	Epigast. reg. Left Right	Hypog. reg. Left Right			Epig. reg. Left	Epig. reg. Right	Hypog. reg. Left	Hypog. reg. Right
43	1600	♀	Injected were 0.3 cc. portions of B. coli com. filtrate	Injected were 0.3 cc. portions of 0.1% solutions of	Ch.	16 mg. Ch.	−	−	−	−
44	1600	,,			Prot. I	24 mg. Prot. I	++	++	−	+
45	1560	♂			Prot. II	24 mg. Prot. II	±	++	±	−
46	1600	♀			Nucleic adids	16 mg. Nucleic acids	−	−	−	−
47	1800	♂			Itinsulfuric acid	24 mg. Itinsulfuric acid	−	−	−	−

TABLE IV

Behaviour of Blood Pressure in Carotic Artery of Pregnant Rabbits, when the Products were injected into Auricular Vein

Product injected	Rabbit No.	Rabbit Weight	Blood pressure (mm. Hg) Before inj.	Time after inj. (min.) 1/2	1	2	3
Ch.	48	1900	82.8	83.5	88	84	85
Protein I	49	2300	84	85	85	83	85
Protein II	50	2500	102	110	111	107	110
Nucleic acids	51	1700	93	98	98	99	103
Itinsulfuric acid	52	1800	81	83.5	85	85	88.5

an itinsulfuric acid) were separated from placentas. Examined physically and chemically, the preparations proved to be mostly contaminated more or less.

2. Schultz-Dale assay did not afford a proof of the presence in the sera of pregnancy-toxemic women of antibodies of those components.

3. Precipitin test suggested the presence of antibodies of the products in some of the sera of normal-pregnant women and more of the sera of the pregnancy-toxemic.

4. The products gave rise to no spasm in the retinal arterioles nor change of intravascular pressure of the carotic artery nor albuminuria

in pregnant rabbits, but all of them excepting Protein II caused premature delivery.

5. Pregnant rabbits underwent pathological lesions of liver and kidney resembling those in pregnancy toxemia by intravenous injection of the preparations, the degree of change depending on the preparation, although Rössle's characteristic allergic changes were not found in those tissues.

6. Proteins I and II proved Schwartzman-active.

Conclusion

The placental components are considered to be causative of pregnancy toxemia partially at least, although it is difficult to decide which are the most effective among them from the present experimental facts.

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Studies on High-molecular Placental Components in Connection with Pregnancy Toxemia

Second Paper

On Purified Placental Neutral Carbohydrate*

By

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The neutral-carbohydrate preparation described in the First Paper[1a] was somewhat contaminated by the itinsulfuric acid, nucleic acids and proteins. Hence, a similar product was further purified to subject again to the biological tests.

The substance obtained which was electrophoretically homogeneous comprised the same sugars as the one before, but contained less of nitrogen, hexosamine and pentose and more of galactose and mannose. Biological examinations gave following results. 1) The skin reaction occurred more markedly in cancer patients than in normal-pregnant, pregnancy-toxemic and non-pregnant women, while in the latter groups of individuals the reaction showed no difference among themselves. 2) After injection into the auricular vein of pregnant rabbits, it caused liver cloudy swelling, degeneration, thrombosis and stasis of blood stream but no necrosis nor necrobiosis, and kidney almost no histological change. When injected into the portal vein it damaged the tissues more evidently. Thus it was revealed to be as causative of pathological changes as the crude material. 3) It gave the positive precipitin reaction with sera of some of pregnancy-toxemic women and of seemingly less of the normal-pregnant, but the negative with the sera of non-pregnant women and of men, suggesting some connection between pregnancy and the placental carbohydrate. 4) The assays if it causes high blood pressure, spasm of central arterioles of retina, albuminuria and premature delivery after injection into pregnant rabbits and Schultz-Dale and Schwartzman reactions re-

* The 170th report of Masamune and co-workers "Biochemical Studies on Carbohydrates." Read before the 5th General Meeting of the Japanese Obstetrical and Gynecological Society, May 10–12, 1953, Niigata[1].

sulted all in negative nearly resembling those in the crude substance. In short, the carbohydrate might be one of the placental components inducing toxemia, but appears to play only a minor etiological rôle.

Experimental

Purification of Placental Neutral Carbohydrate

A crude product prepared as reported[1a] was exhausted with the sat. $(NH_4)_2SO_4$, and the solution was dialyzed, distilled in vacuo to a small volume and precipitated with alcohol. The substance dried was dissolved in as much water as to obtain a 1% solution and drop by drop of 10% lead acetate was added until no more precipitate occurred. Centrifuged. The supernatant of pH 5.8 was bubbled with H_2S gas and then after expelling off of the excessive H_2S by aeration, filtered in a funnel through a double layer of talc and active charcoal repeatedly under suction. The clear filtrate (pH 4.6) was neutralized with 1% NaOH and distilled in vacuo. Some precipitate appeared during the condensation which was removed. The fluid was further distilled to a thick solution and precipitated with alcohol and dried. The substance was again taken up in 100 weights of water and the sat. $Ba(OH)_2$ was added to pH 9.0, whereby a deposit occurred. The centrifuged supernatant was next acidified to pH 2.0 with 1% HCl and the deposit here was also centrifuged off. Shift of the pH by the aid of $Ba(OH)_2$ and HCl was repeated with rejection of occurring precipitates and finally barium was removed with an equivalent quantity of H_2SO_4. Then the solution was electrodialyzed, followed by condensation and precipitation with alcohol. The substance was dried to a constant weight in a desiccator over $CaCl_2$. A white powder was given. The average yield per 100 mg. of the crude material started with was 33 mg. Batches were prepared and combined.

Physical and Chemical Properties of the Preparation

Test-tube tests. Biuret, Goldschmiedt, Neuberg-Saneyoshi, Dische (for desoxypentose) and the direct Osaki-Turumi negative. No glycogen (iodine test) nor phosphorus nor purine was detected. Positive were Molisch (strong), Bial (moderate) and the indirect Osaki-Turumi (faint).

Electrophoresis. As is shown in Fig. 1, only one boundary was demonstrated after electrophoresis of it at pH 7.8.

Paper partition chromatography of the sugar components. 37 mg. of the substance were hydrolyzed with 3.7 cc. of 1 *N* H_2SO_4 in an ampoule by heating at 100°C for 7 hours. The hydrolysate was removed from most of H_2SO_4 and condensed to 0.3 cc., and after adjustment of the pH to

Fig. 1. Electrophoretic pattern of the purified carbohydrate. 30 mg. of the substance were dissolved in 4 cc. of phosphate buffer of pH 7.8 and I 0.29, and examined in a Tiselius apparatus (temperature 10°C, current 15 mA, exposure 80 minutes). The ascending limb to the left. Mobility: the ascending boundary, $\frac{0.3\times0.013308\times0.44}{0.015\times4800}$ $=2.4$ cm^2·volt^{-1}·sec^{-1}·10^5; the descending boundary, $\frac{0.3\times0.013308\times0.42}{0.015\times4800}=2.3$ cm^2· volt^{-1}·sec^{-1}·10^5.

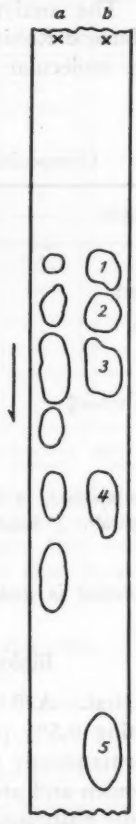

Fig. 2. Sugar chromatogram of the purified carbohydrate diagrammatically reproduced. Irrigation descending. Solvent butanol-pyridine-water (5 : 3 : 2). Temperature and duration of test 25°C and 24 hrs. respectively. Indicator aniline hydrogen phthalate of Partridge. The standard solution for the reference run was 1% with respect to individual sugars, and 0.025 cc. was sampled.
a: main run, b: reference run. 1: chondrosamine hydrochloride, 2: glucosamine hydrochloride, 3: galactose, 4: mannose, 5: rhamnose.

5.8 with H_2SO_4, 0.035 cc. of it was sampled. The chromatogram was developed according to Masamune & Yosizawa[2] as in the case of the crude carbohydrate. Spots were given at the positions corresponding to chondrosamine, glucosamine, galactose, glucose, mannose and a pentose (Fig. 2).

Analysis. The analytical figures are embodied in Table I. The iodine use (Macleod-Robison[6]) amounted to 4.0% as expressed as glucose, wherefrom the molecular weight is calculated at 4500.

TABLE I

Composition of the Purified Carbohydrate

Analysis	In per cent	In equivalents per equivalent weight
N*	2.4	
Hexosamine†	11.9	1.0
Galactose‡	36.8	3.1
Mannose‡	8.4	0.71
Glucose‡	9.0	0.76
Pentose as xylose§	8.2	0.83
Acetyl//	2.5	0.88
Ash¶	3.4	

* Micro Kjeldahl, † Hamasato & Akakura, ‡ Masamune & Ogawa[3], § Masamune & Ogawa[4], // Suzuki[5], ¶ Pregl method without use of H_2SO_4.

Optical rotation in water. $[\alpha]_D^{17}=\frac{0.351\times 1}{0.0156\times 1}=+22.5°$.

Biological Assays of the Preparation

1) Skin test. A 0.05% solution of the substance in physiological saline containing 0.5% phenol was prepared and 0.1 cc. portions were injected intracutaneously to non-pregnant, normal-pregnant and pregnancy-toxemic women and uterus cancer patients, and the shortest and longest diameters of the rednesses were measured after 4 hours. As is illustrated in Fig. 3, the reddened areas (expressed in averages of the two diameters above) were generally bigger in cancer patients than in the remaining groups of individuals, among which no discernible difference of the area was observed.

2) Precipitin reaction. A 2% solution of the substance in physiological saline was diluted progressively with the saline solution, and layed over sera of non-pregnant, normal-pregnant and pregnancy-toxemic women. The occurrence of precipitin reaction was judged after 4 hours. The result is shown in Table II. In some of the sera of the pregnancy-toxemic women the precipitin reaction definitely occurred, and the test resulted in positive also in some of the sera of the normal-pregnant, though apparently less in number. The sera of non-pregnant women and of men gave no reaction. The reaction probably has a connection with pregnan-

cy particularly pregnancy toxemia, but not with severity of the toxemia (Comp. the consequences of the reaction with symptoms of the patients).

3) Pathological changes in liver and kidney of pregnant rabbits to which the substance had been injected. 5 or 10 mg. per kg. body weight of the substance were dissolved in 3 cc. of physiological saline and injected to pregnant rabbits and the animals were killed after 24 hours in the cases of injection into auricular vein and after 48 hours in the cases of injection into portal vein to examine the liver and kidney microscopically. The findings are illustrated in Table III.

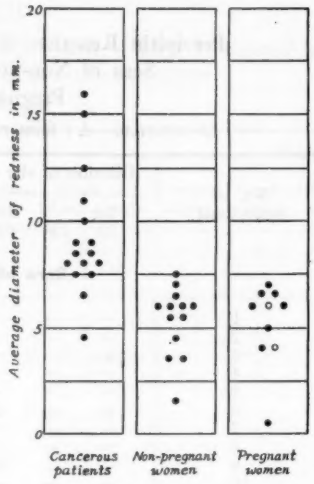

Fig. 3. Showing the results of the skin test with the purified carbohydrate. Open circles indicate reddened areas in pregnancy-toxemic women.

4) In pregnant rabbits were also examined if the substance causes any of high blood pressure, albuminuria and spasm of central arterioles of retina. 5 mg. per kg. body weight of it in 3 cc. of physiological saline were injected into the auricular vein. The methods were same as those described in the First Paper.[1]

a) Blood pressure. Neither increase nor decrease took place as in an example below.

Rabbit		Blood pressure (mm. Hg)				
No.	Body weight (g.)	Before inj.	Time (minutes) after injection			
			1/2	1	2	5
62	2200	115	115	117	117	114

b) No spasm of the central artery of retina was seen after 30 minutes and after 24 hours from the injection.

c) No albuminuria occurred.

To add, no premature delivery happened in all the experiments with the substance on pregnant rabbits.

5) Schultz-Dale reaction. Here 3 cc. of the blood serum of a pregnancy-toxemic woman were intraperitoneally injected into a guinea pig weighing 250–300 g., and after 24 or 48 hours, the animal was killed by bloodletting. An intestine portion was hung in the Tyrode solution to observe the effect of the carbohydrate on its peristalsis (Regarding the details of the method, see the First Paper). Addition of the substance

TABLE II

Precipitin Reaction of the Purified Carbohydrate with the Sera of Non-pregnant, Normal-pregnant and Pregnancy-toxemic Women

− : no reaction, ± : faint r., + : slight r.

No. of individuals	Dilution of the substance (1 :)					Symptoms of patients		
	5×10	10^2	1.5×10^2	2×10^2	∞	Blood pressure (mm. Hg)	Albuminuria	Edema
Sera of non-pregnant women								
1	−	−	−	−	−			
2	−	−	−	−	−			
3	±	−	−	−	−			
4	−	−	−	−	−			
5	−	−	−	−	−			
6	±	±	±	±	±			
7	−	−	−	−	−			
8	−	−	−	−	−			
9	−	−	−	−	−			
10	−	−	−	−	−			
11	−	−	−	−	−			
12	±	±	±	±	±			
13	−	−	−	−	−			
14	−	−	−	−	−			
Sera of normal-pregnant women								
15	±	−	−	−	−			
16	−	−	−	−	−			
17	+	±	−	−	−			
18	−	−	−	−	−			
19	−	−	−	−	−			
20	−	−	−	−	−			
21	+	−	−	−	−			
22	−	−	−	−	−			
23	−	−	−	−	−			
24	−	−	−	−	−			
25	±	−	−	−	−			
26	−	−	−	−	−			
27	−	−	−	−	−			
Sera of pregnancy-toxemic women								
28	++	+	±	−	−	145– 75	(+)	(++)
29	+	±	−	−	−	128– 72	(++)	(−)
30	−	−	−	−	−	162–120	(+)	(+)
31	−	−	−	−	−	140– 90	(+)	(++)
32	−	−	−	−	−	190–100	(++)	(++)
33	±	−	−	−	−	186–120	(++)	(+)
34	+	−	−	−	−	162–110	(−)	(++)
35	−	−	−	−	−	130– 78	(++)	(−)
36	−	−	−	−	−	160–100	(−)	(++)
37	±	−	−	−	−	200–142	(++)	(+)
38*	−	−	−	−	−	170–135	(+)	(+)

* Eclampsia.

Table III

Pathological Lesions in Liver and Kidney of Rabbits of Late Pregnancy after Intravenous Injection of the Purified Carbohydrate

− : no change, + : slight change, ++ : marked change.

Dosage (mg.) per kg. body weight	Rabbit No.	Rabbit Body weight (g.)	Liver						Kidney		Premature delivery
			Necrosis	Necrobiosis	Cloudy swelling	Degeneration	Stasis of blood stream	Thrombosis	Nephrosis	Glomerulonephrosis	
5	53	2,850	−	−	+	+	−	+	±	−	−
	54	2,450	−	−	±	−	−	−	−	−	−
	55	2,320	−	−	±	−	−	±	±	−	−
	56*	2,700	++	±	++	++	+	+	+	±	−
10	57	2,400	−	±	++	+	+	+	+	±	−

* Injected into portal vein.

Table IV

Schwartzman Reaction

− : no change, ++ : marked hemorrhage with or without necrosis of the center of swelling, ## : intense infiltration and swelling of the injected area with necrosis and hemorrhage.

Rabbit Body weight (g.)	Sex	Preparatory inj. Epigast. reg. Left	Preparatory inj. Epigast. reg. Right	Preparatory inj. Hypog. reg. Left	Preparatory inj. Hypog. reg. Right	Reacting inj.	Judgement Epig. reg. Left	Judgement Epig. reg. Right	Judgement Hypog. reg. Left	Judgement Hypog. reg. Right
A. Skin-preparatory potency of the purified carbohydrate										
2,000	♀	0.3 cc. portions of B. coli com. filtrate intracut. inj.		0.3 cc. portions of 0.1% solution of the substance in physiol. saline intracut. inj.		1 cc. of the bacterial filtrate intrav. inj.	++	++	−	−
2,300	♀						The animal died			
2,100	♂						##	##	−	−
B. Reacting potency of the purified carbohydrate										
2,100	♀	〃		〃		24 mg. of the substance* intrav. inj.	−	−	−	−

* Injected as a 1% solution in physiological saline.

(5–10 mg.) to the fluid in which the tissue was suspended resulted in no recognizable contraction of the tissue.

6) Schwartzman phenomenon. The procedure described in the First Paper was applied. In the assay of the reacting potency of the substance 24 mg. of it were intravenously injected. As is illustrated in Table IV, the carbohydrate showed no preparatory potency nor the reacting.

Conclusion

The placental carbohydrate can be one of the etiologic factors of pregnancy toxemia, though subsidiary.

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Studies on Brain Stem

VIII. On the Positions of Abducens Nucleus and Abducens Root of Birds

By

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According to the neurobiotaxis theory by A. Kappers,[1][2] the facial nucleus shifts from a dorsal to a ventral, and the abducens nucleus from a ventral to a dorsal, positions in the course of phylogenetical evolution. We have pointed out the inacceptablity of the above theory in our previous Studies on Brain Stem V in the case of the facial nuclei in birds. In this report we will publish the results of our study on the abducens nucleus in reference with the Kappers' theory.

Kappers[3] has also reported the following interesting observation on the abducens nerve, namely, that he found most of the root fibres of the abducens nerve in a cassowary emerge from the brain stem at a level more to the caudal than the level where the facial root fibres come out, and asserted that it was in consonance with Mesdag's observation (1909) that a certain portion of the abducens nerve emerge at an utterly caudal level in an embryonic chick. Sanders[4] followed with the discovery of some of the abducens rootlets coming out at a more caudal level than the most caudal of the facial nerve fibres in the brain of sparrows.

In view of such a situation, we also tried to investigate the positional situation of the abducens root.

As our materials of study, we used the series of Pal-carmine stained transverse sections of the brains of pigeon, japanese bush warbler, baby crow, gull, owl and cock in store at our laboratory.

Experimental

In all the above enumerated birds, the abducens nuclei were found buried in the reticular substance in a position very close and to the lateral side of the medial longitudinal fasciculus at a dorsal position quite similar to the case of mammalia, but the nuclei, in the case of birds, were always entirely buried in the reticular substance and never appeared above it

to enter the floor of the fourth ventricle. Fuse[5] has divided the abducens nuclei in mammalia into the part submerged in the reticular substance and that jutting into the floor of the ventricle, but in birds, the latter part of the abducens nucleus is not observable. As Fuse has announced these two parts as clearly distinct in cytoarchitecture, it follows that the absence of the latter of the two above parts in a bird's abducens nucleus does not imply its more ventral position. In fact, the position of the abducens nucleus in an avian brain is the same dorsal one as in any mammal. This fact was recognized by Kappers himself, when he stated that the position of the abducens nuclei remains ventral only in a small minority of birds, while in the majority it is on the dorsal side.

Kappers says that the abducens nucleus shifts to a cranial position from a more caudal one in its embryonic development, and in phylogenetical evolution, this shifting takes place more primitively. Bok[6] also endorses this view in his study on chick embryos.

In our study, we found that the abducens nucleus in a pigeon, a japanese bush warbler, a baby crow and a gull appears at a more caudal level than the facial nucleus and disappears also more caudally, while in an owl the abducens nucleus begins to appear more cranially than the facial nucleus and extends to more cranial levels after the facial nucleus has disappeared. In a cock, the two nuclei began and ended at approximately the same levels, the abducens being a bit more on the caudal, if anything. But these observations show only the relative position of the abducens nucleus versus the facial nucleus and do not imply that the absolute position of the abducens nucleus in a bird is more caudal than that in a mammal.

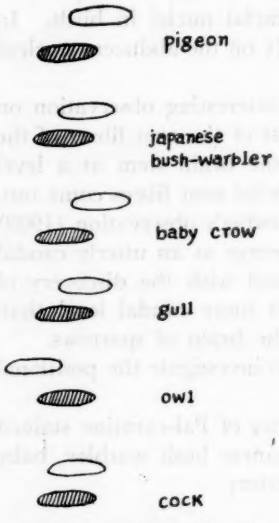

Fig. 1. Topographic relations of the abducens and facial nucleus in a pigeon, a japanese bush warbler, a baby crow, a gull, an owl and a cock. Abducens nuclei are left clear and facial nuclei are designated by stripes.

According to the tenets of the neurobiotaxis theory, it is hard to explain why the abducens nucleus in birds is more caudally situated relative to the facial nucleus, than in mammalia, notwithstanding the fact that the former occupy the same entirely dorsal position in birds as in mammalia. Also it is quite impossible to explain why in such a bird as an owl the position of the abducens nucleus is exactly the same as in mammalia

by means of the neurobiotaxis theory alone.

As the abducens nucleus is more caudally situated than the facial in most birds, the relative position of the abducens root versus the facial root is found to be similar. Upon examining the slides from the caudal side upward to the cranial, in a pigeon, a japanese bush warbler, a baby crow and a gull, the abducens root appears earlier than the facial root and disappears also while the latter is still visible, that is, at a more caudal level than the latter; in an owl, on the other hand, the facial root is situated more caudally, as is the facial nucleus itself. In a cock, the abducens root becomes visible slightly more caudally, and the two roots disappear almost simultaneously, the facial vanishing from sight a bit earlier.

As described above, in birds the abducens root is located more caudally than the facial as a rule, though exceptions such as the owl are not lacking. Consequently, in the order of the cranial nerves, the two exchange places, and the observations of Kappers on cassowary and of Sanders on sparrow are in consonance with our own observations on other birds, in a general way. This fact seems to state a common characteristic of avian brains.

Summary

1. As the results of our study on the brain stems of a pigeon, a japanese bush warbler, a baby crow, a gull, an owl and a cock, we have found that their abducens nuclei were situated entirely dorsally, in a position not much different from those in mammalia.

2. With the exception of an owl, the abducens nucleus is located more caudally than the facial nucleus.

3. The abducens root is also found in a more caudal position than the facial root, with the same exception of the owl.

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On Heat Resistance of *Genus Candida*. A Method Proposed for Pathogenic Discrimination of *Candida albicans*

By

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With the popularization of antibiotics, candidiasis has become a rather common phenomenon recently. However, in such cases, the so-called pathogenic strains merely means the candida strains isolated from foci or excreta etc. of patients, and no reliable method to distinguish them from saprophytic strains has ever been discovered.[1-3] In the course of investigating the heat resistance of *genus Candida*, we found that there is a clear dissimilarity in this respect between the strains removed from pathological foci and those not so removed. But we have to notice that the types of strains available for our tests were too limited in number for final decision, so that we herewith make a tentative proposal subject to further criticisms.

Experimental

Materials. As shown in Table I, five strains of *Candida albicans* isolated from foci or pathogenic products of the diseased kept at the Bacteriological Institute, Tokyo University, one strain isolated from a skin focus at the Dermatological Department, Juntendo University, six strains of the same isolated from faeces from persons in normal health at the Bacteriological Institute of the same University, and seven strains of several species of *genus Candida* donated by Duke University, U.S.A. to Juntendo University, i.e. 19 different strains in total were experimented upon. We herewith express our gratitude due to Prof. T. Akiba of Tokyo Univ., Prof. T. Tsuchiya and Prof. Y. Takahashi of Juntendo Univ. for their kindness in making these strains available for us.

Method. The strains cultured on slanting surfaces of Sabouraud's glucose medium for 7–10 days in an incubator kept at 27°C were transplanted on the same medium in five test-tubes each and immediately heated in a water bath for a fixed duration at a prescribed temperature.

TABLE I

Candida Strains used for Experiment

Strains		Sources	Kept in cultivation at
No.	1	sputum of candidiasis generalisata patient	The Bacteriol. Inst. of Tokyo Univ.
	2	sputum of pulmonary candidiasis patient	"
	3	bile of candidiasis generalisate patient	"
	4	scale of blastomycosis cutis	"
	5	thrush membrane of adult patient	"
Candida albicans	6	scale of blastomycosis cutis	The Dermatol. Dept. of Juntendo Univ.
	13	fæces of person in normal health	The Bacteriol. Inst. of Juntendo Univ.
	47	"	"
	65	"	"
	67	"	"
	85	"	"
	107	"	"
	7078	Duke University U.S.A.	The Dermatol. Dept. of Juntendo Univ.
	7491	"	"
C. Gulliermondi		"	"
C. tropicalis		"	"
C. parakrusei		"	"
C. stellatoidea		"	"
C. psedotropicalis		"	"

A thermometer was placed in a similar medium during the heating and when this indicated the scheduled temperature, the measurement of temperature and time was started. After the scheduled heating was over, the test-tubes were inserted in an incubator kept at 27°C and observed for three days. When a film of candida was formed in three days, it was taken to be viable, and marked +. When no colony grew in three days, the condensed water was smeared on the slant and stood for another three days, and when no colony formed, the strain was assumed to have died out and marked—.

Results. 1) Upon three min. heating (Table II): *Candida albicans* No. 1, 2, 4 and 6 showed viability to the temperature 57°C but died when raised to 58°C. No. 3 and 5 survived the temperature 58°C, but not 59°C. No. 13, 47, 65 and 85 lived up to 55°C and died when heated to 56°C, while No. 67 and 107 were viable up to 54°C but could not stand the temperature above 55°C. Similarly, *Candida stellatoidea* showed viability after being heated to 54°C, *Candida albicans* No. 7078 and 7491, *Candida tropicalis* and *Candida qseudotropicalis* up to 55°C, *Candida Guilliermondi* up to 56°C and *Candida parakrusei* up to 57°C, respectively, but died out when higher temperature was applied.

2) Upon five min. heating (Table III): The maximum temperature

TABLE II

Heated for 3 Minutes

	Strains	Heoted at						
		530°	540°	550°	560°	57°	58°	59°
Candida albicans	No. 1	+	+	+	+	+	−	−
	2	+	+	+	+	+	−	−
	3	+	+	+	+	+	+	−
	4	+	+	+	+	+	−	−
	5	+	+	+	+	+	+	−
	6	+	+	+	+	+	−	−
	13	+	+	+	−	−	−	−
	47	+	+	+	−	−	−	−
	65	+	+	+	−	−	−	−
	67	+	+	−	−	−	−	−
	85	+	+	+	−	−	−	−
	107	+	+	−	−	−	−	−
	7078	+	+	+	−	−	−	−
	7491	+	+	+	−	−	−	−
	C. Guilliermondi	+	+	+	+	−	−	−
	C. tropicalis	+	+	+	−	−	−	−
	C. parakrusei	+	+	+	+	+	−	−
	C. stellatoidea	+	+	−	−	−	−	−
	C. pseudotropicalis	+	+	+	−	−	−	−

TABLE III

Heated for 5 Minutes

	Strain	Heated at						
		53°	54°	55°	56°	57°	58°	59°
Candida albicans	No. 1	+	+	+	+	−	−	−
	2	+	+	+	+	−	−	−
	3	+	+	+	+	+	+	−
	4	+	+	+	+	+	−	−
	5	+	+	+	+	+	+	−
	6	+	+	+	+	+	−	−
	13	+	+	−	−	−	−	−
	47	+	+	−	−	−	−	−
	65	+	+	−	−	−	−	−
	67	+	+	−	−	−	−	−
	85	+	+	−	−	−	−	−
	107	+	+	−	−	−	−	−
	7078	+	+	−	−	−	−	−
	7491	+	+	−	−	−	−	−
	C. Guilliermondi	+	+	+	+	−	−	−
	C. tropicalis	+	+	−	−	−	−	−
	C. parakrusei	+	+	+	+	−	−	−
	C. stellatoidea	+	+	−	−	−	−	−
	C. pseudotropicalis	+	+	−	−	−	−	−

resisted by *C. albicans* No. 1 and 2 was 56°C, by No. 4 and 6, 57°C, by No. 3 and 5, 58°C, by No. 13, 47, 65, 85 and 107 the lower 54°C, by *C. albicans* No. 7078 and 7491, *C. tropicalis*, *C. pseudotropicalis* and *C. stellato-*

TABLE IV
Heated for 10 Minutes

	Strains		Heated at 50°	51°	52°	53°	54°	55°	56°	57°
Candida albicans	No.	1	+	+	+	+	+	+	−	−
		2	+	+	+	+	+	+	−	−
		3	+	+	+	+	+	+	+	−
		4	+	+	+	+	+	+	−	−
		5	+	+	+	+	+	+	−	−
		6	+	+	+	+	+	+	−	−
		13	+	+	+	+	−	−	−	−
		47	+	+	+	+	−	−	−	−
		65	+	+	+	+	−	−	−	−
		67	+	+	+	−	−	−	−	−
		85	+	+	+	+	−	−	−	−
		107	+	+	+	+	−	−	−	−
		7078	+	+	+	+	−	−	−	−
		7491	+	+	+	−	−	−	−	−
	C. Guilliermondi		+	+	+	+	+	−	−	−
	C. tropicalis		+	+	+	+	−	−	−	−
	C. parakrusei		+	+	+	+	+	+	−	−
	C. stellatoidea		+	+	+	+	−	−	−	−
	C. pseudotropicalis		+	+	+	+	−	−	−	−

TABLE V
Heated for 30 Minutes

	Strains		Heated at 50°	51°	52°	53°	54°	55°	56°	57°
Candida albicans	No.	1	+	+	+	+	−	−	−	−
		2	+	+	+	+	−	−	−	−
		3	+	+	+	+	+	−	−	−
		4	+	+	+	−	−	−	−	−
		5	+	+	+	+	−	−	−	−
		6	+	+	+	+	−	−	−	−
		13	+	+	−	−	−	−	−	−
		47	+	+	−	−	−	−	−	−
		65	+	+	−	−	−	−	−	−
		67	+	+	+	−	−	−	−	−
		85	+	+	−	−	−	−	−	−
		107	+	+	−	−	−	−	−	−
		7078	+	+	−	−	−	−	−	−
		7491	+	+	−	−	−	−	−	−
	C. Guilliermondi		+	+	+	−	−	−	−	−
	C. tropicallis		+	+	+	−	−	−	−	−
	C. parakrusei		+	+	+	+	−	−	−	−
	C. stellatoidea		+	+	−	−	−	−	−	−
	C. pseudotropicalis		+	+	−	−	−	−	−	−

idea 54°C and by *C. parakrusei* 56°C, respectively, higher temperatures being fatal in all cases.

3) Upon 10 min. heating (Table IV): The highest permissible

temperature was 55°C by *C. albicans* No. 1, 2, 4, 5 and 6, 56°C by ditto No. 3, 53°C by No. 13, 47, 65, 85 and 107, 52°C by No. 67, 53°C by *C. albicans* No. 7078, *C. tropicalis*, *C. stellatoidea* and *C. pseudotropicalis*, 52°C by *C. albicans* No. 7491, 54°C by *C. Guilliermondi* and 55°C by *C. parakrusei*.

4) Upon 30 min. heating (Table V): The highest temperature survived by *C. albicans* No. 1, 2, 5 and 6 was 53°C, by No. 4 52°C, by No. 3 54°C and by No. 13, 47, 65, 85 and 107 51°C, by No. 67 52°C, by No. 7078 and 7491, by *C. stellatoidea* and *C. pseudotropicalis* 51°C, *C. Guilliermondi* and *C. tropicalis* 52°C and by *C. parakrusei* 53°C.

Summary and Discussion

Upon comparing the strain No. 1 through 6 of *C. albicans* obtained from diseased cases and those isolated from faeces of persons in normal health No. 13, 47, 65, 85 and 107, we find that the former can resist the temperature of 57–58°C and the latter never beyond 54–55°C for three min., showing a difference of more than 1°C in heat resistance. *C. albicans* No. 7078 and 7491 fell under the latter type and of the other species *C. tropicalis*, *C. pseudotropicalis* and *C. stellatoidea* also came in the same cathegory, but *C. parakrusei* showed similarity to the former group in heat resistance and *C. Guilliermondi* occupied a middling position between the two types. Upon five and ten minutes' heating, the same grouping is even more marked but when the heating is kept up for 30 min., the result becomes rather confused and no conclusion can be deduced therefrom. So, by determining the heat resistance of the strain by heating in a water bath for five to ten min., it seems possible to distinguish between strains of *C. albicans* obtained from pathogenic materials and those isolated from normal persons. On this point of view the strains No. 7078 and 7491 of *C. albicans*, the source of which is unknown, may be taken as saprophytic in origin. As for the other species, we had only a single strain each available for our experiment, so it is premature to give final judgment, but we may justified to regard them as saprophytic in origin, except *C. Guilliermondi* which always hovers between the two groups and *C. parakrusei* which shows similarity to the pathogenic group in heat resistance. These two last have been reported as being pathogenic species isolated from endocarditis patient,[1-b)2)] so that we are tempted to see same principle applicable also in the difference in heat resistance by pathogenic and saprophytic strains in other species of *Candida* other than *C. albicans*.

On the other hand, it must be born in mind that the thermal death point of bacteria is apt to "skip" and show undefinable results by meausrement. Williams[3)] upon studying the cause of such skipping, introduced the concept of "basic heat resistance," and Sakaguchi and

Amo,[4] in their study of heat resistance of bacterial spores, have adopted this concept. Our experiments were carried out with due attention to both of these studies, and we have proceeded to make experiments, so to speak, by suddenly heating vegetable cells forming bacterial masses under high humidity.

In any case, at least in the cases of *C. albicans*, we believe we can clearly discriminate between *C. albicans* strains of pathogenic origin from those of saprophytic origin by the established correlation of pathogenity and higher heat resistance, since the saprophytic strains die out upon heating for five min. at 55–56-C or for ten min. at 54–55-C, while the pathogenic strains survive the heating. Since, however, the number of the strains available for our experiments was so limited that we are afraid that our experiment may not prove conclusive.

We must herewith mention with due appreciation that the above experiment was made possible by the allocation of Fund for Scientific Research by the Education Ministry.

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Innervation especially Sensory Innervation of Stomach in White Rat

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Many histological studies on the innervation of stomach have been conducted from olden times, among which the study on the vegetative nerves made by Stöhr[1] during the years of 1931 through 1937, with reference to human normal and diseased stomachs, especially stomachs with ulcus ventriculi, and the recent study of sensory nerves in human stomachs of adults and tenth month embryos by Sato[2] may be especially mentioned as achieving excellent results and contributing honorable pages to the study of the innervation of stomach.

As a humble successor to the illustrious forerunners, I presume to announce my individual views on the subject as stated hereunder, gained after a minute observation of a multitude of ideal preparations of stomachs of white rats, stained with Seto's silver impregnantion.

Individual Views

Vegetative nerves in stomach

By the successive announcement of epochal novel views on the peripheral vegetative nerves as the results of devoted studies by many rising investigators succeeding the renowned studies by Stöhr[1] and Reiser[3] in 1932, an almost entirely new page has been turned in their histology. For example, the end-apparatus of vegetative nerve fibres is no more represented by free terminations, but as the vegetative terminalreticulum (Stöhr), while nothing could be observed to endorse Langley's neuron theory. Also my observations on the distribution of the vegetative nerves and their histological pictures in the stomach in white rat revealed nothing essentially different from those in human stomach. Let me give some detailed views thereon.

The providing nerves for the stomach originate in n. vagus and sympathetic nerves, and upon reaching the curvatura minor, distribute both

ventrally and dorsally with many branches. Then, they run through the outer muscular layer and come in close relation with the Auerbach's plexus formed between the outer and inner muscle layers. In the tela submucosa is found the Meissner's plexus which anastomoses with the minute branches from Auerbach's plexus at many points.

Though in the cross sections of stomach, the Auerbach's pleuxs shows only a nervous distirubtion limited over a very small sphere, in the plane sections it is noticed as a remarkably conspicuous nerve net with rhomboid meshes and containing nerve cell groups. The Meissner's pleuxs which is composed of nerve elements distributed in the tela submucosa is developed best in the outermost layer of the tela and has a little amount of nerve cells in places, as described below.

To go into details of the nerve cells, in the cross sections of the stomach wall are found many nerve cell groups in the Auerbach's as well as the Meissner's plexus. By the latter, the nerve cells are located chiefly in the deeper layer of the tela submucosa, decreasing more and more as the superficial layer is reached, where they are sparsely found in solitary existence.

The nerve cells in these two plexuses are in a stage of development in comparably lower than those in human stomach, as shown in Figs. 1 and 2. For example, the number of their short processes is so far

Fig. 1. Nerve cells in Auerbach's plexus showing low stage of development. Stomach of white rat. Seto's impregnation., X700, reduced to 1/2.

smaller than that in human stomach, that it is impossible to classify these nerve cells into the Dogiel's two types of cells divided by the distribution of the short processes. But there is no doubt about their being sympathetic, because they have been confirmed to be multipolar. In human stomach and intestinal walls, there are also found such extremely lowly differentiated nerve cells on many occasions, and it is very interesting to collate this fact to the data observed by the white rats described above.

Fig. 2. Multipolar nerve cells seen in Meissner's plexus in stomach of white rat. Same staining, X700, reduced to 2/3.

The nerve cells are provided with many mantle cells around them, but no connective tissue capsules can be found around the latter, so that many nerve cells are surrounded by the mantle cells syncytially, as do those in human stomach. These mantle cells are embryologically Kohn's accessory cells and it is surmised they are of endocrine nature.

The vegetative nerve fibres distributed in the stomach are composed of parasympathetic fibres of n. vagus, sympathetic fibres from the sympathetic nerves and long processes from the intramural multipolar nerve cells of sympathetic nature in the stomach. Now the incoming parasympathetic and sympathetic fibres anastomose nervously many times before entering the stomach, so that it is impossible to distinguish these both fibres histologically. The long processes of the nerve cells in the stomach gradually turn into minute fibres as they run farther from the mother cells and become indistinguishable from the incoming nerve fibres.

The providing vegetative nerve fibres first pass into the Auerbach's plexus, wherefrom ramifications of various sizes branch out into both the outer and inner muscle layer, which again send out more minute branches and diffuse into the muscle layers. The branches which run into the tela submucosa pass into the Meissner's plexus. The nerve bundles forming this plexus grow gradually minute as they approach the mucous membrane and passing through the lamina muscularis mucosae finally run into the lamina propria.

The terminal mode of the vegetative nerve fibres consist in forming the terminalreticulum (Stöhr). This terminalreticulum is not represented as free termination as deduced from Langley's neuron theory, but is always connected with each other in endless anastomosis. This terminalreticulum shows a net-like formation of indivisibly fine neurofibrils running cordwise, accompanying neuroplasm-mass interspersed with Schwann's nuclei. The manner of the innervation of the terminalreticulum for all the tissue cells is effected in most cases by contact, in no case entering the protoplasm of the cells.

On the ground mentioned above the terminalreticulum is of sympathetic as well as of parasympathetic nature and furthermore includes the nerve elements of long processes from the nerve cells in the stomach wall, which are probably of sympathetic nature. In consequence, the mechanism of stimuli transmission is deduced to be as follows. In the case when the centrum is excited sympathetically, in the periphery the Schwann's cells in the terminalreticulum are stimulated sympathetically and adrenalin is secreted, while if the central stimulus is parasympathetic, they are stimulated parasympathetically and acetylcholine is produced, these secretions deciding the functions of the innervated cells.

It is understood that, in the terminalreticulum formed to the nerve cells in the stomach wall, the stimuli actuate endocrine secretion in the mantle cells around the nerve cells, the endocrine substances stimulate in turn the nerve cells secondarily and the stimuli are thence transmitted to the periphery by way of the long processes.

Sensory nerves in stomach

Even in the stomach wall of such a comparatively low class mammal as a white rat I could ascertain the existence of sensory nerve elements, i.e. sensory nerve fibres and their terminations. So it would seem too natural that Sato[2] has recently found that such is the case in adult men and tenth month human embryos. He made it clear that the pain in stomach by gastritis, gastric ulcer and presence of parasites is caused by the existence of cerebrospinal sensory nerve elements in the stomach, not by the sensory transmission by autonomic nerves, as advocated by Müller[4] (1924), Kure and Okinaka,[5] for explanation of apains in stomach by postulating existence of nerve fibres to transmit sensory stimuli belonging to vegetative nerve system, to account for their failure to find typical sensory nerve terminations in the stomach. I support Sato's sensory nerve theory on the strength of my discovery of such terminations in the stomach in white rat.

The sensory nerve fibres running in the stomach of the white rat are also provided with conspicuous medullary sheaths as in the human stomach, and represented by axons thicker many times than those of the non-medullated vegetative fibres. Therefore it is very easy to distinguish both of them histologically. These sensory nerve fibres are assumed to originate in n. vagus, as clarified also by the fact that lately Russel[6] succeeded in removing the pain in human stomach caused by gastric ulcer by cutting the vagal nerve.

Now, the sensory nerve fibres which run forward in the providing vegetative nerve bundles advance into the stomach wall from the curvatura

minor and come to the inner muscular layer and other peripheries after passing through the Auerbach's plexus, as shown in Fig. 3. Their terminations are mainly found in the inner muscular layer as well as in the submucosa, and in rarer cases between the submucosa and the lamina muscularis mucosae. But the sensory terminations in the mucous muscular layer were not found by man, and by white rat they are very inferior in number.

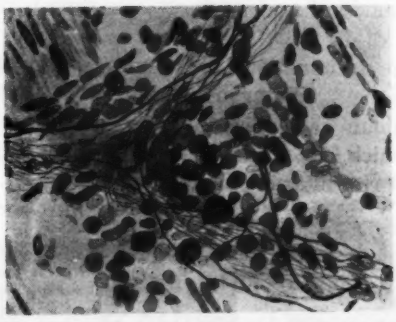


Fig. 3. Thick sensory nerve fibres mingled with thin vegetative nerve fibres in Auerbach's plexus. Stomach of white rat. Same staining, X500, reduced to 2/3.

These sensory terminations are never present in large number, but seemed to be a little more abundant in comparison with those in human stomach. Besides, also in white rat as well as in man the sensory nerve elements are more sparsely found in the corpus ventriculi than in the pars pylorica and pars cardiaca.

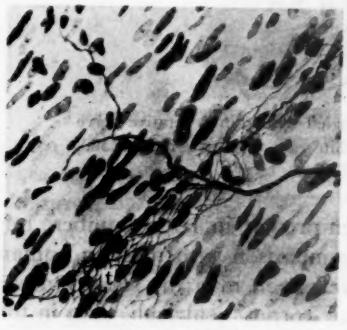
Sada[7] found extremely peculiar sensory terminations existing in the Auerbach's and Meissner's plexus in the human oesophagus. So, Sato[2] expected to find similar terminations in human stomach, but he found only sensory fibres passing through the Auerbach's plexus and no terminations therein. I also could find no sensory nerve terminations in the plexuses in the stomach of white rat.

The ending mode of sensory nerve fibres in the stomach in white rat seems to be divided into the three types of simple branched termination, snake-like termination and unbranched termination. The simple branched terminations are chiefly found in the muscular layers especially in the inner one. This fact is in agreement with the Sato's views in human stomach. As shown in Fig. 4, a thick medullated fibre running through the connective tissue between the muscle bundles in the muscular layer first loses the medullary sheath, then bifurcates two or three times, each branch showing change in size and a contorted course peculiar to the

sensory terminal fibres, finally to terminate sharply or bluntly. But I could not find nodular formations in the tip of the terminal fibres as seen in man. Besides, these fibres also accompany peculiar nuclei, corresponding to Schwann's nuclei, in their course.

Sato[2] has found, in a very small number, simple and complicated glomerular terminations in the human stomach, but I found none of them in the muscular layer of the stomach in white rat. It may be said that the terminal mode of sensory fibres is in the rat more primitive than in man.

The snake-like terminations are found in the tela submucosa and resemble strongly the " Schlingenendigungen " found by Sada[7] in the submucosa of human oesophagus. They are represented by sensory terminal fibres which are comparatively thinner fibres showing little change in size and run in snake-like windings (Fig. 5). They differ from



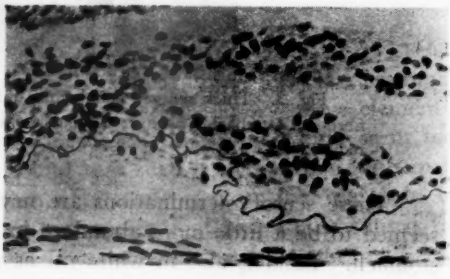


Fig. 4. Simple branced sensory termination seen in the inner muscular layer of stomach of white rat. t vegetative terminalreticulum. Same staining, X500, reduced to 2/3.

Fig. 5. Snake-like termination found in tela submucosa of stomach of white rat. Same staining, X500, reduced to 2/3.

Sada's " Schlingenendigungen " only in having no side branches. These snake-like terminations were not found in the tela submucosa of human stomach (Sato).[2]

The unbranched terminations exist in general in the submucosa and lamina muscularis mucosae. Sato[2] fails to report such terminations of simplest type in the human stomach, but it is probable that they exist also there. At any rate, these terminations consist in that the terminal fibres end sharply or bluntly after a weak winding course, without branching, as shown in Fig. 6.

As stated above, I have ascertained the existence of cerebrospinal sensory fibres and their terminations in the stomach in white rat, though

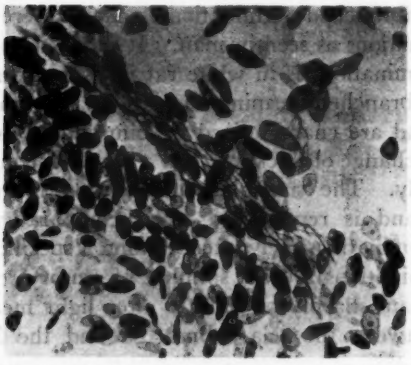


Fig. 6. Non-branched sensory termination running into lamina propria through lamina muscularis mucosae accompanied by vegetative terminal-reticulum. Stomach of white rat. Same staining, X500, reduced to 1/2.

in a small quantity, as in the human stomach, and on this discovery I base my assertion that the transmission of sensory stimuli in the stomach relies on the sensory fibres of cerebrospinal nature in agreement with Sato[2] and not on the vegetative nerve fibres.

Summary

The nerve cells in the stomach in white rat show a stage of development so low that they cannot be divided into any of Dogiel's two types of cells. But, as they are multipolar, they are probably of sympathetic nature.

It is impossible to distinguish histologically sympathetic and parasympathetic nerve fibres as well as long processes of the nerve cells in stomach wall especially at their peripheral parts, because they are in mutual anastomosis at many points. The termination of these vegetative fibres is represented by the terminalreticulum (Stöhr). So the terminal-reticulum must be sympathetic and parasympathetic at the same time. The manner of its nerve supply of the tissue cells is effected mostly by contact.

Sensory nerve fibres and their terminations were found also in the stomach in white rat as in the human stomach (Sato). This fact shows evidently that the transmission of sensory stimuli is done also in the stomach through the cerebrospinal sensory nerve fibres.

The sensory fibres are very easily distinguishable histologically from vegetative fibres. Their number is very small, as is the case in human stomach (Sto).

The sensory terminations may be divided into simple branched,

snake-like and unbranched terminations. There were found no simple glomerular terminations as seen in man. It shows that the differentiation of the sensory terminations is in white rat inferior to that in man.

The simple branched terminations are mainly found in the inner muscular layer and are characterized by bifurcated branches of the stem fibre which show change of thickness, run a winding course and terminate sharply or bluntly. The snake-like termination is generally found in tela submucosa and is represented by a comparatively thin, scarcely branched fibre terminating freely after running a specific snake-like course. Unbranched terminations are the simplest of sensory terminations, where fine fibres terminate sharply or bluntly after light meandering, and are mainly found between the tela submucosa and the lamina muscularis mucosae.

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Innervation especially Sensory Innervation of Duodenum in White Rat

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Many a histological study on the innervation of the stomach and intestine walls has been conducted from olden times, but due to the imperfection of staining methods, the results were tainted with defects. In 1932, the epochal discovery of the terminal formation of the vegetative nerve fibres, that is the terminalreticulum, was made by Stöhr[1] and Reiser,[2] after a painstaking labor. Subsequent observations by many investigators on the subject led to a remarkable illustration of the problem.

On the other hand, the sensory nerves in these parts have hitherto been entirely passed over, perhaps owing to the inveterate notion that no such nerves are found in these regions. However, in the latest days, it has been ascertained that sensory nerve fibres do exist in these parts by Seto[3] and Sato[4] in our laboratory.

In succession to the above mentioned discovery of sensory nerves, I have been able to ascertain the existence of sensory nerves in the duodenum as well as the stomach of the white rat. On the latter I have reported my findings previously.[5] I will make a statement on the innervation of duodenum of the white rat, with special emphasis of sensory nerves, hereunder.

The materials of study were fixed, for a long period, in 10% neutral formol, and cut into 40μ frozen sections, which were stained by Seto's silver impregnantion. Then, the preparations stained very beautifully were subjected to minute microscopic observation.

Individual Views

Vegetative nerves in the duodenum of white rat

The fine structure of vegetative nerves in the duodenum is not different in substance from that in the stomach, as previously reported. No observation was made that would substantiate the traditional neuron

theory, but only the formation of a great reticular nervous system was remarked. Let me outline my views thereon in the following.

The entering nerves into the duodenum derive from the vagal and sympathetic nerves and are in nervous anastomosis at many points on their way. Upon reaching the adventitia of the duodenum, they run inwards from the outer muscular layer and come into close relation to the Auerbach's plexus between the two muscular layers. From this plexus, many minute branches are projected, which come into anastomosis with the Meissner's plexus in the tela submucosa in many points.

The Auerbach's plexus is diffused over a comparatively large area between the inner and outer muscular layers in the cross section of the duodenum. In plane sections, it is seen as a conspicuous rhomboid plexus, with groups of nerve cells here and there, as it is the case with the stomach.

The Meissner's plexus is in remarkable growth in the tela submucosa, especially in its outermost layer, and is provided with many groups of nerve cells in it. The distribution is somewhat different from that in the stomach in white rat, as the nerve cells are disposed in all parts of the submucosa in a diffused state.

The Auerbach's and Meissner's plexuses are well developed but the nerve cells themselves are in a very low stage of evolution, as shown in Fig. 1, as it was also the case in the stomach, and their morphological classification into the two Dogiel's cell types is impossible. But they are probably sympathetic in their nature, because they are almost all multipolar, like those in the stomach.

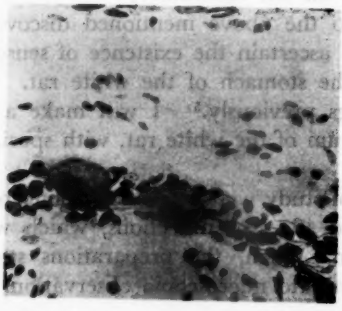

Fig. 1. Two infantile nerve cells seen in Meissner's plexus of white rat duodenum. Seto's silver impregnation, X500, reduced to 2/3.

The syncytial mantle cell layer around the nerve cells is not surrounded with a connective tissue capsule. That is, many nerve cells are found buried in common syncytial mantle cell layers. These mantle cells originate embryologically in the Kohn's accessory cells. Seeing that

cells originating in the accessory cells show endocrine nature without exception, these mantle cells undoubtedly have endocrine functions too.

The vegetative nerves in the duodenum, similar to those in the stomach, consist of parasympathetic fibres from the vagus, sympathetic fibres from the sympathetic nerves and long processes from the multipolar nerve cells in the duodenal wall. As described hereunder, sensory nerve fibres also come into the duodenum (Fig. 2), which are easily distinguished

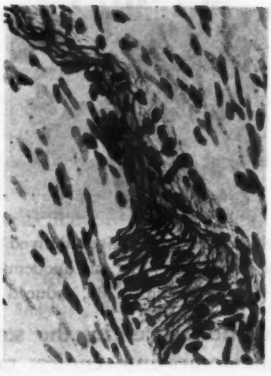

Fig. 2. Many thick sensory nerve fibres running through Auerbach's plexus in white rat duodenum. Thin fibres are vegetative fibres. Same staining, X500, reduced to 2/3.

from the vegetative nerve fibres, due to their mutually different peculiar histological nature, while the histological distinction of the sympathetic and parasympathetic nerve fibres is impossible, as the two types of fibres come into many mutual anastomoses and ramifications, as in the case of the stomach. The long processes of the intramural nerve cells are also undistinguishable from the two types of vegetative fibres above, at the periphery.

In the muscular layers, nerve bundles in various sizes are found to occur, which derive from the Auerbach's plexus standing in close relation with the entering vegetative nerves, but they also do not end freely but always come into mutual anastomosis and finally run into the terminal reticulum. On the other hand, some of the branches from the Auerbach's plexus go over into the Meissner's plexus that is in diffused formation in the tela submucosa. The Meissner's plexus is developed less as it approaches the mucous membrane but gives forth many minute branches thence to the lamina muscularis mucosae and lamina propria mucosae.

The terminal formation of the vegetative nerve fibres is also here represented by the cord-like prolonged terminalreticulum (Stöhr) con_

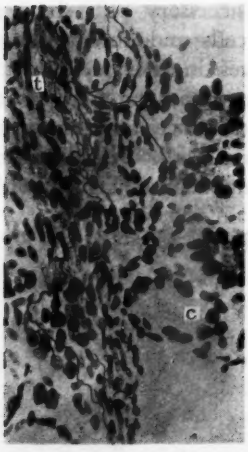

Fig. 3. Branched sensory terminations derived from thick medullated fibres found in lamina propria of white rat duodenum. c intestinal crypt; t terminalreticulum. Same staining, X280, reduced to 2/3.

sisting of minutest neurofibrils, as in the stomach, (Figs. 3 and 5). This never ends free, but comes into endless mutual anastomosis. The terminalreticulum in the duodenum is especially in marked growth in the muscular layers and the tela submucosa and the innervation by contact of the smooth muscle fibres and gland cells is very conspicuous. The terminalreticulum to the nerve cell group does not run cord-wise but is represented as a network consisting of slightly thicker neuro-fibrils and disposed in the whole area of nerve cell group, surrounding many nerve cells commonly. Its histological image is seen as rather indistinct and in a far lower stage of development in comparison with the human cases.

As may be readily seen from the above description, the terminalreticulum of the vegetative nerve fibres is both sympathetic and parasympathetic at the same time. Thus, in the case of sympathetic stimulus given at the nerve center, the Schwann's cells in the terminalreticulum, which are suposed to be endocrine, secrete adrenalin in response to the sympathetic stimulus, while when a parasympathetic stimulus comes in, acethylcholin is secreted, and these secretions will determine the functions of the innervated cells. This is the same as I have reported on the nerves in stomach.

Sensory nerves in white rat duodenum

Seto[3] has found a considerable number of sensory nerve fibres originating in the nn. splanchnici running into the duodenum by tenth month

human embryo and ascertained their terminal formations. He is under the notion that these nerve fibres play an important part in the reflex secretion of digestive juice normally, and in pathological cases, the pain sensation in the duodenum.

In continuance of his study, I could ascertain the existence of conspicuous sensory nerve fibres and their endings in the duodenum in white rat, a kind of mammlas of comparatively lower evolution. Thus it is beyond doubt that the sensory transmission in the duodenum is dependent on the cerebro-spinal sensory nerve fibres, as well as it is the case in the stomch, where the existence of sensory fibres in the wall has been ascertained previously. Consequently the traditional positulate of sensory transmission by vegetative nerve fibres is doomed to natural death. I wish to assert in particular that the pain sensation in stomach and duodenum has nothing to do with any autonomic nerve fibres of a so-called sensory nature as postulated by Müller,[6] Kure and Okinaka.[7]

My views on the existence of sensory nerve fibres and their endings in the duodenum in white rat are as follows. Since the sensory nerve fibres are characterised by the considerable thick axon-cylinder and the having of the medullar sheath, they may be quite easily distinguished from non-medullated thin vegetative nerve fibres and the transit of the sensory fibres into the duodenum is very distinct. These are probably originated in the splanchnic nerves running in the dorsal abdominal walls, advancing into the adventitia of the duodenum near the caput pancreatis, in bundles of various sizes, together with a small number of vegetative nerve fibres. Thence they run into the Auerbach's plexus, as shown in Fig. 2, and further into the tela submucosa or into the lamina propria mucosae, to terminate there.

Contrary to the case of white rat stomach,[5] here in the duodenum I found almost no terminal formation of sensory nerve fibres in the muscular layers, as by the human tenth month embryo studied by Seto.[3] Sada[8] reports existence of sensory terminations of very peculiar form in the ganglia of the Auerbach's and Meissner's plexuses in human oesophagus, but I could not find such terminations in the stomach as well as the duodenum in white rat. Here, the sensory fibres merely run through the ganglia of the plexuses without showing any terminal formation.

The terminations of the sensory nerve fibres in the duodenum in white rat are divisible into the following 3 kinds, namely the branched, the glomerular and the snake-like endings. Branched endings are mainly formed in the lamina propria mucosae, though some are found also in the tela submucosa. The other two types of terminations are observable only in the tela submucosa.

According to Seto,[3] in the duodenum of the 10th month human

embryo the sensory nerve fibres all end in branched terminations, and this type was the most prevalent also in the duodenum in white rat. The trunk fibres are sometimes represented by comparatively thick fibres (Figs. 3 and 4) but sometimes by finer fibres (Figs. 5, 6 and 7).

Fig. 4

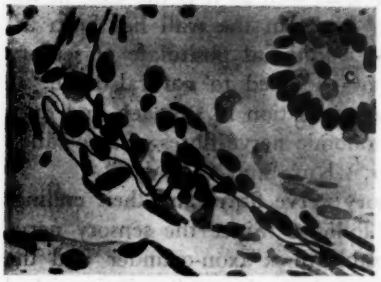

Fig. 5

Fig. 4. A bifurcated sensory termination of a thick nerve fibre and an unbranched termination of a fine nerve fibre found in lamina propria of white rat duodenum. c intestinal crypt. Same staining, X500, reduced to 2/3.

Fig. 5. A bifurcated sensory termination derived from a comparatively thin medullated fibre found in white rat duodenum. c intestinal crypt; t vegetative terminalreticulum. Same staining, X280, reduced to 1/2.

These medullated fibres, large and small, as they come nearer the endings, lose their myelin sheath, and run to the periphery with meanderings peculiar to the sensory terminal fibres, ending, in most cases, in wide diffusion in the lamina propria, after sending out a few branches or in some cases many branches, though not rarely they end as unbranched terminations without branching out at all. The larger fibres show prominent change in size during their course, due to the distention of the fibrils. The terminal branches mostly end sharply, but in some cases bluntly.

In the tela submucosa, the branched endings are found sometimes in the close vicinity of duodenal glands, sometimes in bigger lymphatic nodes extending into the tela submucosa. In the lamina propria the branched endings run around the intestinal crypts, some reaching into the connective tissue of intestinal villi, and some even close up to the intestinal epithelium. But none is found to enter the epithelial cells to pass over into intraepithelial fibres there.

Fig. 3 and Fig. 4 both show branched terminations composed of comparatively thicker fibres formed in the lamina propria mucosae. The sensory termination at the left side of Fig. 3 is a branched ending composed of several branches covering a wide area. The terminal branches show conspicuous change in size at many points and peculiar windings, ending sharply or bluntly. Fig. 4 shows a bifurcated sensory termination derived from a thick sensory fibre accompanying an unbranched termination originating in a fine sensory fibre.

Fig. 5 shows a bifurcated termination composed of minute fibres spreading up to the close vicinity of the intestinal epithelium, and Fig. 6 a simple branched ending derived from a fine sensory fibre around the duodenal glands and an unbranched termination of a fine fibre ending in a solitary lymphatic node in the tela submucosa. In Fig. 7, many sensory nerve fibres, mostly minute, are seen running between the duodenal glands and a lymphatic node in tela submucosa, without showing any marked winding. They are presumed to form a sensory branched termination originating in one trunk fibre.

Fig. 6

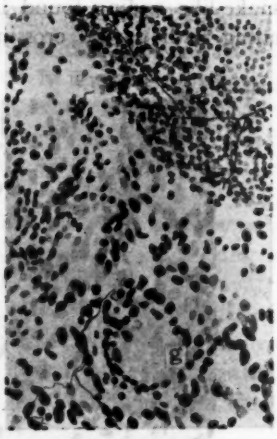

Fig. 7

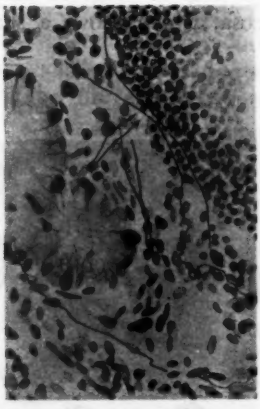

Fig. 6. Branched sensory terminations found in tela submucosa and solitary lymphatic node of white rat duodenum. g duodenal gland. Same staining, X500, reduced to 1/2.

Fig. 7. Many thin sensory nerve fibres found between duodenal glands and a lymphatic node in tela submucosa of white rat duodenum, probably representing a branched termination derived from one main fibre. Same staining, X400, reduced to 2/3.

Though I could not find glomeurlar endings in the stomach of the white rat, seeing that Sato[4] found a few of them in the mucous membrane

of human stomach, I was led to infer their existence in the duodenum of white rat, and after a very painstaking research, could ascertain the existence of this type of endings in the tela submucosa of white rat duodenum, though in very rare instances.

These glomerular endings are composed of comparatively thick fibres, which show considerable change in size and peculiar meandering in their course, to present as the whole a glomerular arrangement of the terminal fibres, as shown in Fig. 8. In the glomerulum specific cell nuclei of syncytial nature are found, but the connective tissue capsule around it is lacking.

The snake-like endings were first discovered by Sada[8] in the human oesophagus, and I also could ascertain their existence in the tela submucosa of the stomach in white rat. Now I found them also in the tela submucosa of duodenum in white rat, and in some cases, in highly typical formation.

These endings consist in sensory nerve fibres of medium size running a typical snake-like or wave-like winding course for a comparatively long interval, after losing their medullar sheath, to end mostly bluntly, as shown in Fig. 9. Fig. 10 also shows a snake-like ending found in tela submucosa, which shows a snake-like winding with partial crossing, re-

Fig. 8

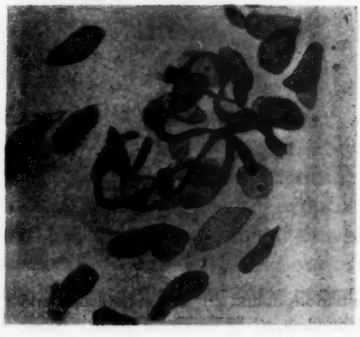

Fig. 9

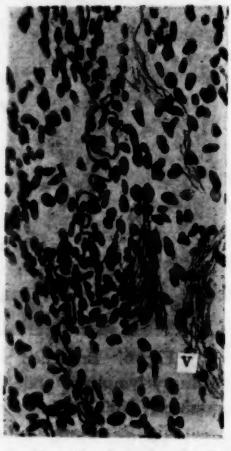

Fig. 8. A non-capsulated simple glomerular termination composed of a thick fibre found in tela submucosa of white rat duodenum. Same staining, X1000, reduced to 1/2.

Fig. 9. A snake-like sensory termination found in tela submucosa of white rat duodenum. v vegetative nerve fibres. Same staining, X420, reduced to 2/3.

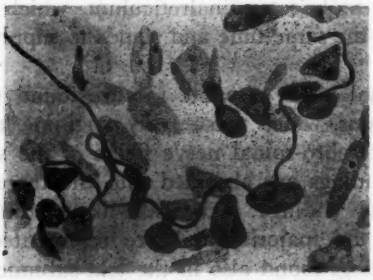

Fig. 10. The same as Fig. 9. Here the termination shows a partial loop-like running course. Same staining, X1000, reduced to 2/3.

presenting an extremely simple glomerular running. Snake-like endings with side branches were seen very rarely, if any, as in the stomach[5] as well as in the duodenum.

Thus I was enabled to ascertain the existence of typically cerebro-spinal centripetal nerve fibres and their free endings in the walls of white rat stomach and next in the walls of its duodenum. This fact is in good agreement with the results of experiments by Dragstedt and Palmer[1] and proves that the sensory transmissions in the stomach and duodenum do not rely upon any autonomic nervous system, beyond all controversy.

The reason why a system of sensory nerve fibres of cerebro-spinal nature exists in the duodenum lies in that it means a formation of a reflex arc physiologically, as is the case with human duodenum, as shown by Seto.[3] So, when the acid substances from the stomach come down into the duodenum the sensory nerve endings in the latter respond to the stimulus, send it to the center and reflexively excite the autonomic secretory efferent fibres to activate the function of the duodenal glands, pancreatic glands, vesica fellea etc., i.e. the secretion of digestive fluids. When the duodenum is in an abnormal, that is, in a diseased state, I concur in the opinion, these nerve endings are the factors that cause the peculiar pain sensations in these regions.

Summary

The intramural nerve cells in the duodenum are very low in development as those in the stomach,[5] by white rats, so that they cannot be classified into the two kinds of Dogiel's Type I and Type II cells. But the fact that they are multipolar seems to indicate that they are sympathetic. As the sympathetic and parasympathetic nerve fibres as well as the peripheral parts of the long nerve processes of the nerve cells in the walls are in anastomosis with each other, they cannot be distinguished histologically. The end apparatus of these vegetative nerve fibres is

represented by the Stöhr's terminalreticulum, which is sympathetic and parasympathetic at the same time and stands to supply all the tissue- cells by contact.

In the duodenal walls of white rat are found sensory nerve fibres and their terminations, which presumably originate in the comparatively abundant sensory cerebro-spinal nerve fibres of the splanchnic nerves.

The sensory endings are classified into the three types of branched endings, glomerular endings and snake-like endings. The branched endings constitute the majority and are represented as comparatively widely diffused endings found also in the tela submucosa, but principally in the lamina propria mucosae. These endings consist of rather large sized main fibres, after losing their myelin, dividing into some branches which show in places neurofibril expansions and peculiar windings, and end sharply or bluntly. In the submucosa, these endings frequently reach the duodenal glands or run into lymphatic nodes. In the propria mucosae, they run around the crypts or further into the villi and even close up to the epithelium.

The glomerular endings are very rarely formed in the submucosa. They consist of thicker nerve fibres showing change in size and winding courses. They have some special syncytial nuclei but lack a connective tissue mantle.

The snake-like endings consist of sensory nerve fibres of medium size, which, upon becoming unmyelinated, run in snake-like or wave-like windings for a comparatively long course and terminate sharply or bluntly. Sometimes, these endings show a partial glomerular course.

The existence of sensory nerve terminations in the duodenum, as described in the above, shows that, here, as well as in the stomach,[5] transmission of sensory stimuli is effected by way of cerebro-spinal nerve fibres. The reason of their existence presumably consists, physiologically, in the formation of a reflex arc to promote secretion of digestive fluids, and pathologically, in the causing of peculiar duodenal pains.

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A Proposal on a Calculation Method to be applied by Geographical Comparison of Cancer Mortality

By

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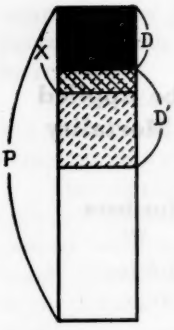
Heretofore, either crude or age-adjusted death rate has been used as the basic figures for geographical comparison of cancer mortality. Crude death rate is subject to influence of the age composition of the population, so that it is quite inappropriate for such a use as it is. In order to correct this defect, age-adjusted death rate has been widely used. However, while trying to compare the cancer mortality of various countries using age-adjusted death rates, we have been convinced that even these rates were not wholly adequate for an equitable comparison. The greatest defect of an age-adjusted death rate is that it is too closely dependent on the age composition of a standard population. For instance, when this rate is used in comparing the cancer mortality of several countries, different results may be obtained by taking the population of one country or another as standard, and sometimes even the order of the magnitude of the cancer mortality may be upset. That is to say, these figures lack absoluteness. In order to get rid of such a defect, some methods of corrections have been proposed, but none of these has proved wholly satisfactory.

In order to obtain numerical figures having a possibly absolute meaning, we have tried a system of computing the cancer mortality modifying the method applied to the preparation of abridged life tables. The method is to find out how many persons out of 100,000 born in a certain district have died from cancer and make geographical comparison according to this supposed number of deaths. As a matter of fact, we get at some different figures by the difference in the method of how to deal with those who have died of other causes except cancer. Hereunder, we will describe one of these methods.

Suppose that there is no cause of death but cancer. First of all, we must establish the cancer death rates by age group. Now, in a certain age group, if we express the number of population by P, the actual number



of cancer deaths by D and the actual number of deaths from other causes than cancer by D', the death rate by cancer in general mean is D/P. If we assume that no death except from cancer occurs, it may be presumed that a part of the actual number of deaths from other causes must have died from cancer. Use X to denote the total number of cancer deaths, i.e., D plus this presumptive number of cancer deaths. If it is assumed that this presumptive number of cancer deaths included in D' occurs in the proportion X/P, then $X=D+D'\cdot(X/P)$, or $X/P=D/(P-D')$. We computed this ratio for each age group.

Next we made computation after the method of calculating the data in abridged life tables. In Table I, "Mortality rate ($_5q_x$)" means the ratio of the number of persons dying for age group x~x+4 to the total number of those living up to the age x, "Number living (l_x)" means the number surviving to the exact age of x, "Number dying ($_5d_x$)" means the number of deaths for age group x~x+4 and "Cumulative number dying (c_{x+4})" the total of deaths between the ages 0 and x+4.
So,

$$_5d_x=l_x\cdot{}_5q_x$$
$$l_{x+5}=l_x-{}_5d_x$$
$${}_5d_{x+5}=l_{x+5}\cdot{}_5q_{x+5}=(l_x-{}_5d_x)\cdot{}_5q_{x+5}$$

We begin the calculation on the assumption that the number living at the age 0 (l_0) is 100,000.

Thus, we can find the number dying for each age group if we know the mortality rate.

The cumulative number dying up to the age of x+4 years is calculated as follows:

$$c_{x+4}={}_5d_0+{}_5d_5+{}_5d_{10}+\cdots\cdots+{}_5d_{x-5}+{}_5d_x$$

Applying the values of X/P multiplied by 5 for ages x~(x+4) years

TABLE I

Age interval x~(x+4) years	Mortality rate $_5q_x$	Number living l_x	Number dying $_5d_x$	Cummulative number dying c_{x+4}
0~ 4	0.000120	100,000	12.0	12.0
5~ 9	0.000040	99,988.0	4.0	16.0
10~14	0.000045	99,984.5	4.5	20.5
⋮	⋮	⋮	⋮	⋮
80~84	0.028340	84,134.1	2,384.4	18,250.3

previously found out to ${}_5q_x$, we have obtained the data given in Table I (${}_5q_x$ in the abridged life table is not calculted using the five-fold value of death rate for ages $x \sim (x+4)$ as it is, but upon correcting it by a rather complicated adjustment. In this paper, however, for simplicity's sake, we have used the five-fold value of death rate by age group as it is).

Table II show the figures calculated by the above mentioned method illustrating the mortality from malignant neoplasm in Japan, England and Wales and U.S.A. in the year 1949. For reference, we have indicated the age-adjusted death rates standardized to the Japanese population. The figures in parentheses stand for the comparative indices with Japan (100) as basis.

TABLE II

	Cumulative number dying up to the age 85	Age-adjusted death rate
Male:		
Japan	18,250 (100)	73.4 (100)
England & Wales	31,221 (171.1)	104.6 (142.5)
U.S.A. (White)	28,095 (153.9)	91.4 (124.5)
U.S.A. (Nonwhite)	21,056 (115.4)	89.4 (121.8)
Female:		
Japan	13,755 (100)	69.0 (100)
England & Wales	22,974 (167.0)	91.9 (133.2)
U.S.A. (White)	22,915 (166.6)	96.6 (140.0)
U.S.A. (Nonwhite)	18,732 (136.2)	101.7 (147.4)

It may be said that this method has a defect in that it involves the unnatural assumption that there is no cause of death other than cancer. Since, however, it is thus saved from being influenced by other causes of death, it is, we believe, the most appropriate for geographically comparing the status of cancer mortality. In this method, it is presumed that a part of those who are classified as died from other causes actually had died from cancer. But as in fact, cancer plays a major role as a cause of death over the other causes of death, we are afraid the values we have calculated are a little too high. It is necessary for this commputation to have the exact numbers of the population and deaths by age group. When the eldest age group is indicated somewhat as "above . . . years of age", we have no means to make computations; in such cases we can only find out the cumulative number dying up to the next lower age group. On the other hand, when we make such computations on cancer mortality by the site of cancer, the problem of how to treat those who died from cancers in other sites than the one in question will present itself. It we calculate the figures assuming that there is no death from cancers in other sites, the total of the figures thus calculated for each site will not add up to the figures computed for cancers in all sites in one.

After all, the figures found out by this method will show how many persons out of 100,000 babies born simultaneously will die in the lapse of time from their birth to a certain year of age, under the assumption that cancer death rates by age group in a year will hold good in subsequent years too and that there is no death from other causes but cancer. Quite different from the age-adjusted death rate system, no standard population ever used in our method and the resultant values do not involve any external element except death from cancer. From this point of view, it can be called a method to insure absolute values indicating the status of cancer mortality.

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Innervation of Duodenum and Ductus Choledochus in Human Embryo

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Considering its biological and clinical importance, the histological study on the innervation of the stomach and intestine walls have been undertaken by many investigators in the past, but due to the difficulty of staining nerve elements, the general picture of the nerves supplying the alimentary canal has not been adequately cleared up to date. For the first time in 1932, utilizing Bielschowsky's silver impregnantion as improved by himself, Stöhr[1] succeeded in making a thoroughgoing study of the nerve system supplying the human stomach and intestine walls, especially in regard to normal and diseased stomachs (gastric ulcer in particular), and made an epochal contribution to the gastric neurology. However, his study was limited to the vegetative nerves in its scope, and no description was given upon afferent nerves. In other words, the histology of nerves in the stomach as far as it concerns with vegetative nerves was brought to a nearly perfected system by his researches, but nothing was reported by him on the sensory nerves and their terminations.

However, as there is no doubt that, beside the normal reflex functions, the stomach and intestine, especially the duodenum, are subject to pain sensations due to diseases, it is easy to infer that sensory nerves should be found in these parts too. In fact, quite recently, Sato[2] (1948) has announced his discovery of sensory nerve terminations in his preparations of human gastric wall stained with Seto's silver impregnation, and Seto[3] himself succeeded in ascertaining that sensory nerve fibres terminated in the duodenum in tenth month human embryo (1949). Ohi[4] followed by reporting his discovery of sensory nerves and their terminations in the stomach and the duodenum in white rat, and Kimura,[5] in 1953, followed up and endorsed the reported results of the above mentioned studies.

Treading in the steps of these forerunners, I had the opportunity to contribute my humble share in the study, with the duodenum and ductus choledochus of fourth and seventh month human embryos as my materials.

These were fixed in 10% neutral formol solution for a long period, cut into 40μ frozen sections and stained with Seto's admirable silver impregnation method. The series of the numerous beautiful preparations thus obtained, I subjected to a minute microscopic study and thus succeeded in arriving at many interesting observations concerning the subject, which I will report in some detail in the following.

Individual Observations

I. The vegetative nerves in the duodenum and the ductus choledochus

As is well known, the vegetative nerves in the duodenum are composed of Auerbach's plexus between the two muscle layers and Meissner's plexus in the submucosa, as is the case with the stomach and the other intestines. In these plexuses, frequent groups of multipolar nerve cells are found, which are classifiable into Dogiel's Type I and Type II cells, according to the arrangement of their nerve processes.

The entering nerves into the duodenum are represented by sympathetic fibres originating in the sympathetic nerve ganglia and parasymsympathetic fibres coming from the vagal nerves. These two kinds of fibres proceed to the Auerbach's plexus, send out numerous nerve rami toward the Meissner's plexus and closely connect with them. The tissue constituting the wall of the duodenum, such as smooth muscle fibres, cells of the duodenal glands, the epithelial cells and others stand under the control of these vegetative nerve fibres, through contact by Stöhr's terminalreticulum.

The above findings are very distinctly observable in human adult duodenum, but nothing has been hitherto reported, as far as I am aware, on the development of the vegetative nerves in the duodenum of embryos. This absence of literature on the subject is probably due to the inadequate delicacy of the staining methods hitherto available in handling the overdelicate nerve fibres in such specimens. My observations obtained by the beautifully stained preparations were as outlined in the following.

These ideally silver-stained slides of mine revealed every detail of the nerve elements, down to the minutest fibrils, in the duodenum of fourth month embryo, with excellent clarity. Especially, in the preparations where the other tissues were stained with good definition, the nerve elements were also particularly distinct.

In fourth month embryo, the nerve cells found in the Auerbach's and Meissner's plexus are composed of a very large cell nucleus and little cytoplasm staining red and containing very ill-developed neurofibrils, and the nerve processes emerging from the cells are scarcely detectable as yet, as shown in Fig. 1. That is to say, the nerve cells in the plexus in the duodenum of fourth month human embryo are not yet in the shape

of typical multipolar cells as in adults, but are merely round cells with a oversized cell nucleus, containing only very minute neurofibril net formed in their cytoplasm in rare cases. The nuclei of the mantle cells are smaller than those of the nerve cells and it was noted with interest that they were very small in number in such an early stage of the embryonic life. This fact seems to indicate that the nerve cells are not subject to further mitosis, while the mantle cells would go through active mitosis in later days.

In seven month embryo, the nerve cells are somewhat larger and well-developed, neurofibrils are sometimes seen in their cytoplasm. Also nerve processes are seen emerging from some of the cells (Fig. 2). However, the number of the nerve processes is much smaller than in adults,

Fig. 1

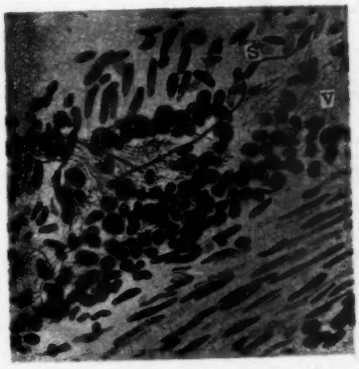

Fig. 2

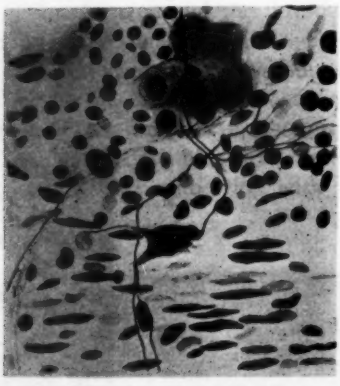

Fig. 1. Auerbach's plexus in the duodenum of a 4th month human embryo. Nerve cells are of infantile type; v fine vegetative fibres; s thick sensory fibres. Seto's silver method. X700, reduced to 1/2.

Fig. 2. Auerbach's plexus in the duodenum of a 7th month human embryo. Fine neurofibrils in the nerve cells and some nerve processes emerging from the nerve cells are observable here and there. Same staining. X650, reduced to 2/3.

so that it is as yet impossible to distinguish the two kinds of Dogiel's Type I and Type II cells. That is to say, even in the seventh month of embryonic life, the nerve cells are as yet largely simple round cells, though some extremely simple multipolar cells are not absent. The mantle cell nuclei are somewhat more numerous than in fourth month embryo, but interestingly enough, they are incomparably fewer than in human adult.

My preparations of the duodenum of the fourth and seventh month embryos also clearly demonstrated that the entering vegetative nerves, both vagal and sympathetic, running into the duodenum ramify and

anastomose many times before reaching the duodenum, as is usual with the vegetative nerves in other parts. The picture of these nerves running through the adventitia into the Auerbach's plexus, which send out numerous rami into the Meissner's plexus connecting very closely with the latter, has been also very clearly seen in my human embryos.

The Meissner's plexus in the duodenum as well as in other parts of the small intestine is much better developed than that in the stomach. It is comparatively evenly developed in the deeper as well as in the superficial layers of the submucosa, and the groups of nerve cells are accordingly found widely scattered in all parts of the submucosa (Fig. 3), though particularly well developed in the most external layer of the submucosa.

Fig. 3. Meissner's plexus provided with nerve cell groups in places and a typical branched sensory termination in the propria of the duodenum. 7th month human embryo. Same staining. X300, reduced to 1/3.

It has been now incontrovertibly established that the terminal apparatus of vegetative nerve fibres is represented by the Stöhr's so-called terminalreticulum. Its embryological development has been brought to light by many studies accomplished at this laboratory, according to which, the terminalreticulum comes to nearly perfect maturation in sundry organs by the fourth month of embryonic life (Yamada,[6] Yabuki[7] and Mizukoshi).[8]

In my fourth month embryos, the terminalreticulum in the duodenum was in remarkable formation, in conformance with the above doctrine, and was even better developed in the seventh month embryo. In par-

ticular, in the muscle layers and around the duodenal glands which become markedly developed in the seventh month, the formation of the terminalreticulum was typically advanced. This terminalreticulum stands in nervous control over the sundry tissue cells through contact by surrounding them. Schwann's nuclei are seen here and there in the terminalreticulum, which I take to be endocrinal in nature, as many of my predecessors have supposed to be. I presume these nuclei secrete adrenalin or acethylcholine as the occasion demands.

Seeing that very little has been written on the vegetative innervation of the choledochus, I will detail hereunder my observations on the matter. In the ostial part of the choledochus adjacent to the duodenum, that is, the most strongly developed part of the choledochus, in my fourth and seventh month embryos, I found to my surprise that the vegetative nerves were as richly provided as in the walls of the stomach and the intestines. In the layer around the choledochus consisting of connective tissue fibres, conspicuous plexus was formed by well-developed vegetative nerve bundles, almost completely surrounding the duct (Fig. 4). In this plexus also groups of vegetative nerve cells were found here and there, though in a smaller number than in the Auerbach's and Meissner's plexus mentioned above (Fig. 5). This plexus is presumedly in close connection with the

Fig. 4

Fig. 5

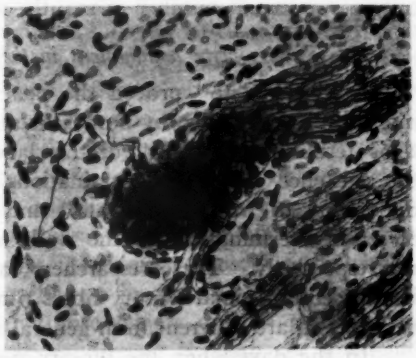

Fig. 4. Distribution of the nerve elements in the ductus choledochus in the 7th month human embryo. p plexus in the periphery; vegetative terminalreticulum (t) and unbranched sensory endings (s) in the propria. Same staining. X150, reduced to 2/5.

Fig. 5. A nerve cell group in the plexus formed in the periphery of the ductus choledochus. 7th month human embryo. Nerve cells are mostly of infantile type. Fine fibres are vegetative and thick fibres are sensory. Same staining, X350, reduced to 2/3.

entering vegetative nerves, i.e., vagal and sympathetic nerve fibres.

From this plexus, numerous nerve branches reach into the subepithelial connective tissue layer of the mucous membrane of the ductus choledochus, then branch out into more numerous and minuter branches, which come into mutual nervous anastomosis at frequent intervals, till the minutest terminal branches or fibres go over into the vegetative terminalreticulum, as shown in Fig. 4. The terminalreticulum diffuses widely in the subepithelial connective tissue layer to control the mucous lands and the epithelium in this part through contact from their circumference. The terminalreticulum is also formed very conspicuously in the comparatively ill-developed smooth muscle layer found at the periphery of the mucous membrane.

The nerve cells in this part, as was the case in the duodenum, are generally round and provided with a large, round nucleus, in my fourth month embryos. In the seventh month embryo, the cell bodies have increased in size, but most of them are as yet mere spheres, as shown in Fig. 5. Some of them, however, show conspicuous formation of neurofibril net in their bodies, and send out two or three nerve processes from their periphery, being represented as simple multipolar cells. So, it is impossible, here too, to distinghish the two kinds of Dogiel's Type I and Type II cells in the embryonic state, as little as in the duodenum. The status of mantle cell nuclei was also similar to that in the duodenum, only a very small number of mantle cell nuclei being found in existence.

II. Sensory nerve terminations in the duodenum and the ductus choledochus

Recently, Seto[3] proved the penetration of the duodenum in tenth month human embryo by numerous sensory fibres and described in detail their terminations. Ohi[4] of this laboratory also succeeded in proving the existence of sensory terminations in the mucous membrane of the duodenum in white rat. Sato[2] and Ohi[4] demonstrated the existence of sensory terminations in the walls of the stomachs of man and white rat respectively. These researchers concluded that these fibres originate in the cerebro-spinal sensory fibres running in the splanchnic or in vagal nerves, and are different from general sensory nerve fibres in their nature.

In the days when the sensory nerves in the stomach and the duodenum were unknown, it was believed that the pains felt in the stomach and the duodenum were perceived by way of some vegetative fibres which partook the afferent nature (Müller,[9] Kure and Okinaka).[10] Now that purely sensory nerve fibres were discovered in these parts as described above, such imaginary interpretations have to be discarded.

I was given the occasion to follow up the existence and the development of sensory fibres and their terminations found by Seto in the duo-

denum of tenth month human embryo in my embryos of four and seven months, and succeeded in establishing further evidence of the existence of sensory fibres in human duodenum, as described hereunder. Their existence was also proven in the choledochal duct where it approaches the ostium. Thus it has been clarified that pain sensations caused by the pathological disturbances in the stomach and duodenum as well as the choledochus, are felt owing to the existence of sensory nerve terminations ending in these parts. My observations are detailed in the following.

Seto[3] has proved the existence of nerve bundles comprising a large number of thick medullated fibres starting from the contact area of the head of the pancreas and the duodenum and running into the latter, and assumed that these thick sensory fibres are derived from the splanchnic nerves reaching the vegetative nerve plexus in the posterior abdominal wall. This description was found to apply to my fourth month and even better to my seventh month embryos. These nerve bundles enter into the adventitia of the duodenum, running longitudianally and gradually dividing into branches, which reach the Auerbach's plexus through the outer muscle layer. The thick sensory fibres among them penetrate further into the submucosa through the inner muscle layer.

Sada[11] has observed plexus-like terminations of sensory fibres widely distributed in the Auerbach's and the Meissner's plexus in human oesophagus, but in the Auerbach's plexus in my preparations of duodenum I could find no such terminations, quite as Ohi has failed to discover such terminations in the stomach and the duodenum in white rat. The sensory terminations distributed in the duodenum of my human embryos were limited to those formed in the submucosa or those of the sensory fibres penetrating into the tunica propria through the muscle layer of the mucous membrane. The medullated fibres entering into the submucosa run either separately or in parallel columns of two or three fibres for some length, then branch out and terminate in the submucosa or in the tunica propria after passing through the lamina muscularis mucosae. These terminal branches often show changes in size and winding courses peculiar to sensory terminal fibres, diffuse widely and in general end sharply. They often reach the positions directly beneath the duodenal glands, especially in my seventh month embryos, and beneath the epithelium of the crypts and the villi, both in my fourth and seventh month embryos, but never go further into the glandular or epithelial cells. In short, the sensory terminations found in the duodenum consist of widely distributed branched terminations in the submucosa and in the mucous membrane.

However, the terminations found in fourth month embryo were

much smaller than those in Seto's tenth month embryo.

Fig. 6 shows a rather complex branched termination formed around the duodenal glands in the submucosa of the duodenum in seventh month human embryo. Its terminal branches are seen to reach the surroundings of the glands, but none of them penetrate further into the glandular cells. Fig. 7 shows a comparatively simple branched ending spreading out with a peculiar winding in the superficial layer of the submucosa or directly beneath the muscularis mucosae, in the duodenum of a seventh month embryo. The terminal fibres show the change in size peculiar to the general terminal sensory fibres.

Fig. 6

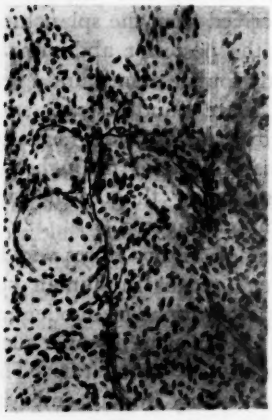

Fig. 7

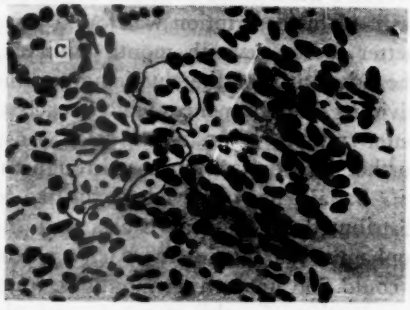

Fig. 6. A branched sensory termination formed in the surroundings of the duodenal glands. 7th month human embryo. Terminal branches end sharply. Same staining. X270, reduced to 2/5.

Fig. 7. A simple branched sensory termination showing a specific winding course formed in the deeper layer of the propria of the duodenum. 7th month human embryo. c crypt. Same staining. X480, reduced to 2/3.

Fig. 8 is an illustration of a typical branched termination of a sensory nerve fibre running from the submucosa through the mucous muscle layer and spreading out widely in the tunica propria in the duodenum of a seventh month human embryo. The terminal branches end in sharp points, some of which reach the surroundings of the crypts but none penetrate further into the epithelium.

In my preparations of the opening part of the ductus choledochus of my human embryos, I found a rather large number of thick sensory fibres coming in. These fibres generally terminate in simple branched terminations in the connective tissue layer of the mucous membrane

provided with mucous glands, but often some unbranched terminations are also observable and their terminal fibres end sharply in general, but never run into glandular or epithelial cells.

In Fig. 4 you may see many thick sensory fibres running in the plexus formed in the connective tissue layer at the periphery of the choledochus of a seventh month human embryo, and two unbranched terminations ending in the mucous gland layer. In Fig. 9 also, several sensory fibres are seen ending in the mucous gland layer, and one simple branched termination is observed beside a few unbranched terminations.

Fig. 8

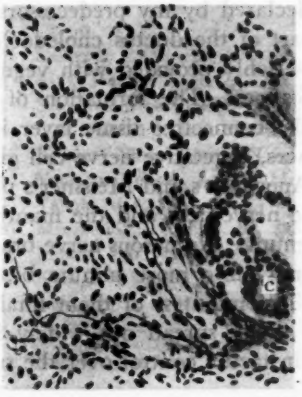

Fig. 9

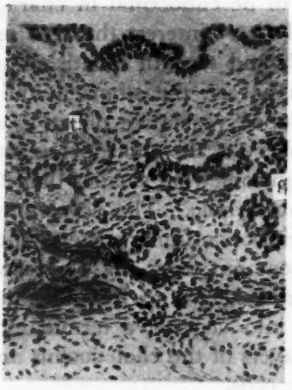

Fig. 8. A typical branched sensory termination distributed in the propria of the duodenum. 7th month human embryo. c crypt. Same staining. X300, reduced to 2/5.

Fig. 9. Thick sensory nerve fibres and their unbranched and simple branche terminations in the propria of the ductus choledochus. 7th month human embryo. t terminalreticulum; g mucous glands. Same staining. X270, reduced to 2/5.

Summary

In the duodenum of fourth month human embryo, the intramural nerve cells show an infantile stage of development represented by spherical cells with a large round nucleus. In the cell bodies neurofibrils are as yet scarcely formed. Mantle cell nuclei are much smaller than the nerve cell nuclei in size and at this stage of embryonic life, are much smaller in number than those in human adults. The mitosis of these cell nuclei is considered to take place gradually in later days.

In seventh month embryo, the nerve cells increase somewhat in size, but the majority is still represented by spherical cells with a large round nucleus, except a minority of the cells, by which neurofibrils and a few

nerve processes begin to appear, showing the formation of incipient simple multipolar cells. Mantle cell nuclei are more numerous than in fourth month embryo but far lesser than in human adult.

The vegetative terminalreticulum is in a nearly perfect formation already in the fourth month on the embryonic life, as has been established by many researches in this laboratory, and also in my specimen of embryonic duodenum, this doctrine has been beautifully confirmed. In the seventh month embryo, the terminalreticulum is even better developed and surrounding the sundry tissue cells enter into tactile control over them. The Schwann's nuclei found in the terminalreticulum I take to be endocrinal in character, as declared by my predecessors.

It is of interest that the opening part of the ductus choledochus of fourth and seventh month embryos is as richly provided with vegetative fibres as are the stomach and the intestines. The formation of well-developed vegetative plexus is seen in the connective tissue layer in the periphery of the choledochus, and in places, vegetative nerve cell groups, though somewhat smaller than those in Auerbach's and Meissner's plexus, are observable. The development of the nerve cells and the mantle cell nuclei is on a similar stage as in the duodenum. Numerous nerve branches are sent out from the plexus, which ramify into more minute branches and anastomose at frequent intervals. The minutest of them finally go over into vegetative terminalreticulum, which spread out widely in all the layers of the choledochus and stand in tactile control over the sundry tissue cells.

I have succeeded in proving the existence of medullated sensory fibres and their terminations not only in the duodenum but also in the choledochus of fourth and seventh month embryos. The pains caused by pathological disturbances in the duodenum and choledochus could be thus attributed to the existence of sensory terminations in these parts.

Large nerve bundles containing numerous sensory fibres which are presumably originated from the splanchnic nerves reach the contact area of the head of the pancreas and the duodenum and thence the major part runs to the duodenum and the rest to the pancreas and the choledochus.

The numerous sensory fibres that reach the adventitia of the duodenum run through the outer muscle layer into the Auerbach's plexus and thence into the submucosa through the inner muscle layer to terminate in the submucosa or further in the mucous membrane. In these layers, the sensory fibres lose their myelin and branch out into rami, which, upon running winding courses with peculiar change in size, spread out widely and terminate sharply. The terminal branches sometimes reach the surroundings of the duodenal glands or the epithelium of the

crypts and villi, but never penetrate into the cells of the glands and epithelium themselves. The above described sensory fibres are of course much better developed in seventh month embryo than in fourth month one.

Many sensory nerve fibres are also seen penetrating into the plexus found in the periphery of the embryonic choledochus. These fibres run also here into the mucous membrane and end in unbranched and simple branched terminations therein. Their terminal fibres end sharply. No fibres are seen penetrating into the cells of the mucous glands or the superficial epithelium to pass over into intraepithelial fibres.

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individuals and that those of placenta give the positive skin test for cancer but not for pregnancy. To add, placental components, which were separated afterwards by a more gentle procedure, have been found to behave in pregnancy-toxemic women dissimilar from the preparations above from the same tissue, as will be published later in this Journal.

In addition, it is worthy to note that the embryonal neutral-carbohydrate-fraction contained no mannose nor glucosamine which must be of significance in embryochemistry.

Experimental

Components of Placenta

They were prepared as already described by Kanno *et al.*[4] Regarding their physical and chemical properties, confer also that article.

Fractions of Embryo

Preparation procedure

Fetus within the first three months of development were placed in 5 volumes of 91% alcohol for a week. They were then dried in a Faust apparatus at 37°C and ground into a powder in a mortar. The weight was found reduced to 1/7–1/8.

50 g. of the material given were shaken with 500 cc. of 0.14 *M* NaCl for 24 hours. The centrifuged supernatant was set aside and the insoluble residue was subjected to a similar extraction five more times. The solutions were united, 10% HCl added to pH 4.5, and after centrifuging off of the deposit (**I**), the supernatant was neutralized and distilled to about 1/10 the volume.

Neutral carbohydrate-fraction. The condensate was at first half-saturated with $(NH_4)_2SO_4$ and the centrifuged supernatant was further full-saturated with the salt and again centrifuged. The supernatant which contained some floating particles was filtered and the filtrate was dialyzed for a week, when it was free from ammonium and sulfate ions. Some deposit that had occurred during dialysis was removed. Followingly the fluid was distilled under reduced pressure to about 20 cc. and 10% lead acetate was added until no more precipitate occurred. The centrifuged supernatant here was bubbled with H_2S-gas and freed from lead sulfide by repeated filtrations through kaolin and charcoal layers on a Toyo Roshi filter paper No. 4 in a funnel. It was next adjusted to pH 9.0 with the sat. $Ba(OH)_2$ and after rejecting the precipitate thereby, quickly acidified to pH 2.0 with 10% HCl, followed by again rejecting the precipitate hereby. Such shift of pH hither to thither was processed again and again to liberate the solution from all the thus precipitable and finally Ba was removed with a just sufficient quantity of 10% H_2SO_4.

Then the solution was electrodialyzed for 24 hours, precipitated with abs. alcohol and dried in vacuo over $CaCl_2$. A white powder was given. 68 mg. was the crop.

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The deposit III was treated with the HCl-containing alcohol and the chloroform-amylalcohol similar to above and the final alcoholic precipitate was also dried—Nucleic acid fraction. Yield 88 mg.

Protein-fraction. The deposit II was taken up in the sat. NaCl and neutralized with 1% NaOH. The insoluble part was discarded and the solution was acidified to pH 4.2 by the aid of 1% HCl to separate the protein or proteins. The precipitate was further purified by repetition of dissolution and precipitation, and when it became only faintly Molisch positive, dissolved over again in water (by the aid of a little alkali) for dialysis, which was continued for 5 days. Finally the solution was distilled in vacuo to a thick solution and dried up in a desiccator over changes of $CaCl_2$. 134 mg. of a grey substance were given.

Chemical properties

Test-tube tests and quantitative analyses. The results are embodied in Table I. All the fractions must have been impure, because the neutral carbohydrate fraction contained much glycogen, the nucleic acid fraction more of N than the amount calculated from the P content (N/P in most pure nucleic acid preparations is 1.7–1.8), the itin and itinsulfuric acid fraction Molisch-giving impurities and the protein fraction, whose carbohydrate content was no more than a trace, fairly less N than ordinary

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On Skin Reaction in Cancer Patients and Pregnant Women of Placental and Embryonal High-molecular Components

By

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Matsubara[1] announced that watery extracts of such proliferating tissues as cancer, chorionic villi and testis produce, when injected intracutaneously, a larger redness in cancerous patients than in the non-cancerous, and in the pregnant women than in the non-pregnant. Later on, Ogawa[2] and Masamune & Ogawa[3] have demonstrated that a glacial acetic acid-soluble portein and a mixture of the polypeptide-polysaccharide complex and the itinsulfuric acid from placentae give the positive skin test for cancer. The present writers have undertaken to examine various kinds of water-soluble high-molecular component of the same organ in the above respect, and furthermore whether or not the rapidly growing embryo possesses such a component or components. Fractionation of placentae was carried out as already reported[4] and that of embryos by a somewhat modified procedure. Of the placental substances (crude), "Itinsulfuric acid" and "Nucleic acids" in particular exhibited a distinctly larger reddened area in cancer (uterus cancer) patients than in pregnant women, including the pregnancy-toxemic (Cf. Kanno *et al.*[4] and Kanno[5] also), and the non-pregnant. Between the latter groups of women almost no difference of the average area was seen. The reactions by the remaining substances, among which "Protein II" probably contained Ogawa's protein, the writers incline to believe, showed similar trend though not evident. Of the embryonal preparations, the neutral carbohydrate-, itin and itinsulfuric acid- and protein-fraction proved to give more intensive reaction in cancer-suffering and pregnant women than in the non-pregnant, and the nucleic acid-fraction possessed the same but lower potency. Thus it looked that most high-molecular substances of embryo have a peculiarity of giving a larger redness in cancerous patients and pregnant women than in non-cancerous, non-pregnant

individuals and that those of placenta give the positive skin test for cancer but not for pregnancy. To add, placental components, which were separated afterwards by a more gentle procedure, have been found to behave in pregnancy-toxemic women dissimilar from the preparations above from the same tissue, as will be published later in this Journal.

In addition, it is worthy to note that the embryonal neutral-carbohydrate-fraction contained no mannose nor glucosamine which must be of significance in embryochemistry.

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Preparation procedure

Fetus within the first three months of development were placed in 5 volumes of 91% alcohol for a week. They were then dried in a Faust apparatus at 37°C and ground into a powder in a mortar. The weight was found reduced to 1/7–1/8.

50 g. of the material given were shaken with 500 cc. of 0.14 *M* NaCl for 24 hours. The centrifuged supernatant was set aside and the insoluble residue was subjected to a similar extraction five more times. The solutions were united, 10% HCl added to pH 4.5, and after centrifuging off of the deposit (**I**), the supernatant was neutralized and distilled to about 1/10 the volume.

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TABLE I

Results of Test-tube Tests and Analyses of the Chemical Fractions of Embryo

Test or analysis	Fractions			
	Neutral carbohydrate	Nucleic acid	Itin and itinsulfuric acid	Protein
Biuret	±	−	−	++
Molisch	++	+	++	±
Goldschmiedt	±	−	++	−
Neuberg-Saneyoshi	±	−	++	−
P	−	++	−	±
Bial for pentose	+	+		
Dische for desoxypentose	−	+		
Purine*	−	+		
Glycogen†	++			
N (%)	2.1	13.7	2.9	12.8
P (%)‡		5.2		
Ash	3.0	21.2	8.7	1.9

* The hydrolysate was tested with an ammoniacaline $AgNO_3$ solution. † Iodine test. ‡ Haneda, Sinokawa and Yasuoka's modification of Plimmer method.

proteins.

Paper partition sugar-chromatography of the neutral carbohydrate. 30 mg. of the substance were sealed together with 3.0 cc. of 1 *N* H_2SO_4 in an ampoule and heated for 7 hours in boiling water. The hydrolysate was freed from most of H_2SO_4 by the aid of the sat. $Ba(OH)_2$ and distilled in vacuo to 0.3 cc., of which a 0.035 cc. portion was sampled for descending irrigation according to Masamune & Yosizawa[6] with butanol-pyridine-water (5 : 3 : 2). Temperature and duration of test 25°C and 24 hours respectively. Indicator aniline hydrogen phthalate of Partridge. In Fig. 1 is reproduced the chromatogram given, which possessed spots of chondosamine, galactose, glucose and a pentose, but lacked those of glucosamine and mannose.

Skin Reaction

The substances were dissolved in 2,000 volumes of physiological saline containing 0.5% phenol. A 0.1 cc. portion of the solutions were injected intracutaneously on the flexor side of forearm and the longest and shortest diameters of the reddened areas were measured by means of a caliper after 4 hours. Those diameters of each redness were summed up and divided by 2. Figs. 2–10 show the average diameters of the rednesses in cancerous (uterus cancer) patients and pregnant (including the pregnancy-toxemic) and non-pregnant women.

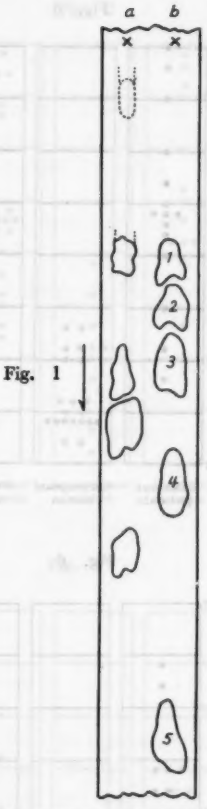


Fig. 1

Fig. 2

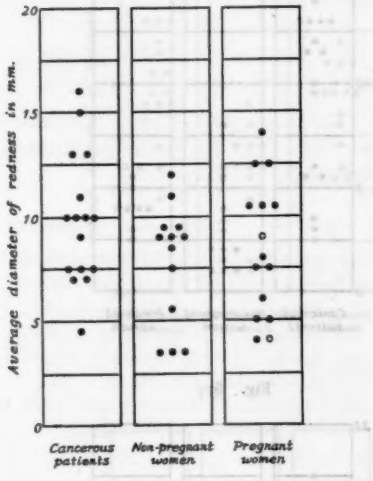


Fig. 1. Diagrammatic reproduction of the chromatogram of a hydrolysate of the embryonal neutral carbohydrate fraction. The standard solution for the reference run was 1% with respect to individual sugars and a 0.035 cc. portion was sampled.

a : main run, b : reference run, 1 : chondrosamine hydrochloride, 2 : glucosamine hydrochloride, 3 : galactose, 4 : mannose, 5 : rhamnose.

Fig. 2. Showing the results of skin test with the placental neutral carbohydrate in cancerous patients and pregnant and non-pregnant women. Open circles indicate reddened areas in pregnancy-toxemic women.

Fig. 3

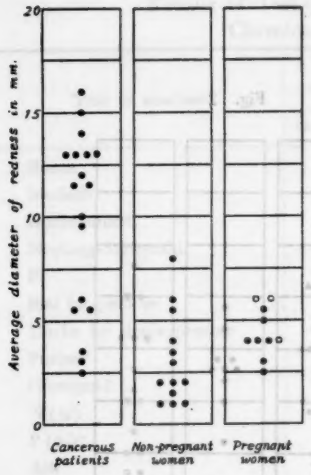


Fig. 4

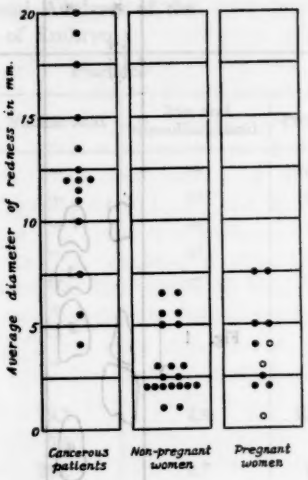


Fig. 5

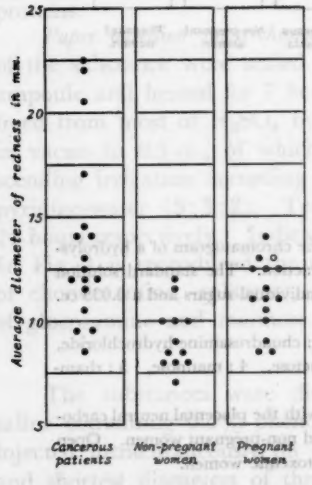
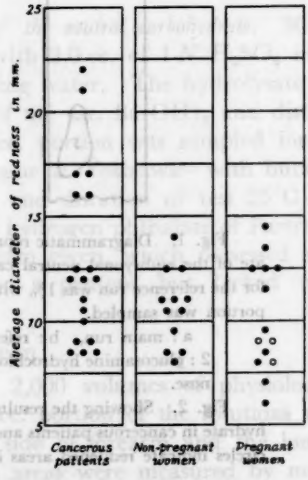


Fig. 6



Figs. 3–6. Showing the results of skin test with the placental itinsulfuric acid (Fig. 3), nucleic acids (Fig. 4) and Proteins I (Fig. 5) and II (Fig. 6) in cancerous patients and pregnant and non-pregnant women. Open cirlces indicate reddened areas in pregnancy-toxemic women.

Fig. 7

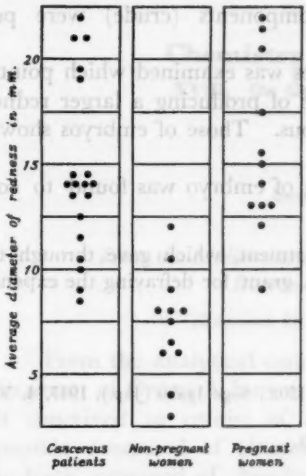


Fig 8

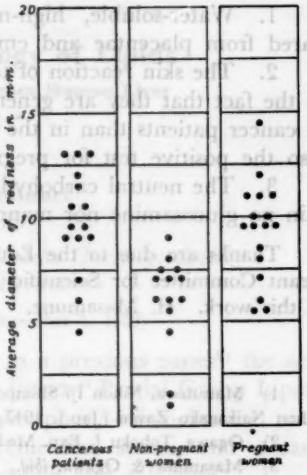


Fig. 9

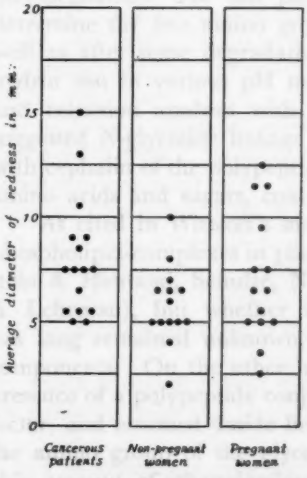
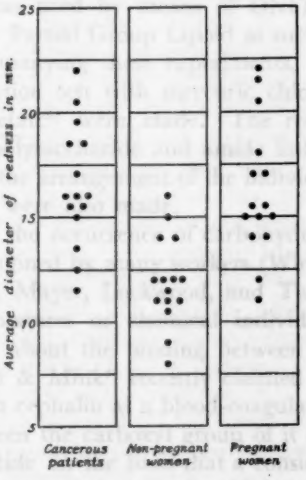


Fig. 10



Figs. 7–10. Showing the results of skin test with the embryonal neutral-carbohydrate-(Fig. 7), itin and itinsulfuric acid-(Fig. 8), nucleic acid-(Fig. 9) and protein-(Fig. 10) fraction in cancerous patients and pregnant and non-pregnant women.

SUMMARY

1. Water-soluble, high-molecular components (crude) were prepared from placentae and embryos.

2. The skin reaction of the substances was examined which pointed to the fact that they are generally capable of producing a larger redness in cancer patients than in the non-cancerous. Those of embryos showed also the positive test for pregnancy.

3. The neutral carbohydrate fraction of embryo was found to contain no glucosamine nor mannose.

Thanks are due to the Education Department, which gave through the Grant Committee for Scientific Researches a grant for defraying the expenses of this work. H. Masamune.

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2) Ogawa, Tohoku J. Exp. Med., 1952, **56**, 185.

3) Masamune & Ogawa, *ibid.*, 1952, **56**, 269.

4) Kanno, Kimura, Tanaka & Mugikura, *ibid.*, this volume, p. 257.

5) Kanno, *ibid.*, this volume, p. 271.

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Chemistry and Biology of Lipids

XVIII. On Group Lipoid from Human Liver

Fifth Report*

By

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From the analytical results reported in a previous paper[2] the active fragment of Group A human liver group lipoid "Partial Group Lipoid" is conceived to consist of acyl-glycerophosphoryl-ethanolamine and a peptide composed of glutamic acid, glycine and serine, and a carbohydrate composed of chondrosamine, mannose and galactose.

The present communication deals with an attempt to disclose the modes of mutual linkage of the groupings cephalin, polypeptide and polysaccharide. For this purpose, it was tried by means of DNFB to determine the free amino groups in the Partial Group Lipoid as such as well as after some degradation. Accompanying these experiments, ninhydrin test in various pH media, reduction test with mercuric chloride and retention analysis with copper acetate[6] were made. The results suggested N-glycoside linkage with the polysaccharide and amide linkage with cephalin of the polypeptide. As to the arrangement of the individual amino acids and sugars, contemplations were also made.

As cited in Wittcoff's monograph,[3] the occurrence of carbohydrate-phospholipid-complexes in plants was imagined by many workers (Winterstein & Hiestand, Schulze, Nottbohm & Mayer, Lockwood, and Taylor & Lehrman), but whether they are mixtures or chemical individuals has long remained unknown, much less about the binding between the components. On the other hand, Hecht & Mink[4] recently claimed the presence of a polypeptide conjugated with cephalin as a blood-coagulating factor, and assumed amide linkage between the carboxyl group of it and the amino group of the glycerophosphatide on the basis that a considerable amount of ethanolamine and serine were recovered after Van Slyke-desamination. Nevertheless, such compound substances as group lipoids

* A part was read before the 25th Annual Meeting of the Japanese Biochemical Society, Tokyo, April 26-28, 1953.[1]

have never before been a subject of study.

Experiments and Comments

Material

Preps. I and II of the Partial Group Lipoid which had been prepared by Masamune *et al.*[2] were employed. The materials were electrophoretically homogeneous and contained α-glycerophosphoric acid, ethanolamine, glutamic acid, serine, glycine, galactose, mannose and chondrosamine in molecular proportions of respectively 2:2:2:1:1:3:2:2 and 2:1:2:1:1:3:2:2 and carboxylic acids. They inhibited isoagglutination of A erythrocytes at a dilution of $1:6.4\times10^3$ but that of B erythrocytes at a dilution of $1:2\times10^3$. Regarding details see the original article.[2]

Free Amino Groups in the Partial Group Lipoid

Changing of the Partial Group Lipoid into the dinitrophenyl (DNP)-derivative

The procedure was principally the same as the one developed by Sanger[5] and co-workers. The run with Prep. I will be described in the following. 50 mg. of it were taken up in 1 cc. of water, adjusting with 0.2 *N* NaOH to neutral, and after addition of 50 mg. of $NaHCO_3$ and 1 cc. of ethanol containing 0.05 cc. of dinitrofluorobenzene (DNFB)[6] (pH of the mixture 8.5), shaken for 2 hours at room temperature (about 5°), whereby the turbid mixture (yellowish brown) became clearer. It was condensed by vacuum-distillation, and 1 cc. of water was added. The solution was freed from excess DNFB by thorough exhaution with ether and then from the ether by bubbling with air, followed by acidification with 1 *N* HCl to a pH lower than 1.2. The flocculent precipitate was centrifuged and washed once with 0.05 *N* HCl, twice with an ether-ethanol mixture (3:1) and once with ether in sequence and dried in a vacuum desiccator over H_2SO_4. Yield, 49 mg. It was more soluble in acetone, ethanol and methanol than the material started with. The isoagglutination inhibition potency was found to have been barely altered.

Analysis of the DNP-Partial Group Lipoid

The qualitative. The DNP-Partial Group Lipoid obtained above was hydrolyzed and DNP-amino acids and -amines set free were extracted and identified paper-chromatographically:—

30 mg. of the DNP-Partial Group Lipoid were suspended in 1 cc. of 6 *N* HCl and refluxed in a micro apparatus at 100° for 4 hours. The hydrolysate was shaken three times with 1 cc. of ether, and the combined ethereal solutions (yellowish) were condensed to a syrup on a water-bath and placed in a vacuum desiccator over solid caustic soda to remove most

of HCl. The residue was dissolved in a few drops of 0.01 *N* NaOH and the slightly alkaline solution was made up to about 0.3 cc. with ethanol, of which 0.05 cc. portions were paper-chromatographed in a descending manner. For the irrigation Blackburn & Lowther's solvents[7] propanol-cyclohexane (1 : 3 by volume), saturated with 0.05 *M* phthalate buffer of pH 6.0, and benzylalcohol-ethanol (9 : 1 by volume), saturated with the same buffer solution, were applied,[8] and spots of DNP-ethanolamine, -glycine and -chondrosamine and probable dinitrophenol (An elongated spot was given between the spots of DNP-glycine and -chondrosamine) were demonstrated. For reference runs, DNP-amino acids were prepared according to Sanger,[5] DNP-glucosamine according to Annison *et al.*[9] and *DNP-ethanolamine* as follows:

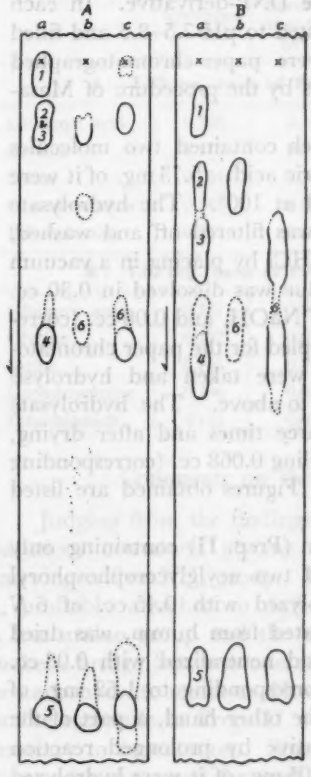

Fig. 1. Chromatograms diagrammatically reproduced of the DNP-amino acid and -amines set free from the DNP-Partial Group Lipoid (Prep. I) by hydrolysis. A : Irrigated with propanol-cyclohexane (1 : 3) saturated with 0.05 *M* phthalate buffer of pH 6.0. B : Irrigated with benzyl-alcohol-ethanol (9 : 1) saturated with 0.05 *M* phthalate buffer of pH 6.0. Distance between the original spots and solvent front 45 cm. Temperature of test 24°.

a : reference run with DNP-amino acids, -glucosamine, and -ethanolamine, b : reference run with an ether extract of the hydrolysate of the DNP-derivative of brain cephalin, c : main run. 1 : DNP-glutamic acid, 2 : DNP-serine, 3 : DNP-glycine, 4 : DNP-glucosamine (DNP-chondrosamine is located at the same position), 5 : DNP-ethanolamine, 6 : probable dinitrophenol.

0.2 g. of ethanolamine hydrochloride[10] was dissolved in 2 cc. of 1 *N* NaOH, shaken together with 50 cc. of 90% ethanol containing 0.5 cc. DNFB (less than

the equivalent) vigorously for a while and stood overnight in an ice chest. The ethanol was evaporated off under reduced pressure at below 10°C, and the remaining solution was acidified with several drops of 1 *N* HCl to pH 1–2 and immediately treated with two changes of ether. The ethereal solutions were combined, distilled to a syrup and placed in an ice chest, and the appearing orange yellow needles were filtered off and dried in a brown vacuum-desiccator. F.P. 87–88°.

N (Friedrich method) 18.7% (Calc. for $C_8H_9O_8N_3$ 18.5%).

In Fig. 1 are diagrammatically reproduced the chromatograms.

The quantitative. A weighed amount of the Partial Group Lipoid and its DNP-derivative were hydrolyzed with HCl, and stood in a vacuum desiccator over NaOH, as such in the case of the Partial Group Lipoid itself and after ether extraction in the case of the DNP-derivative. In each case, the residue was taken up in water, adjusted to pH 7.5–8.5 and filled up to a definite volume, whose portions were paper-chromatographed for the analysis of the ninhydrin-colored spots by the procedure of Masamune *et al.*[2]

Prep. I of the Partial Group Lipoid which contained two molecules each of ethanolamine and acylglycerophosphoric acid. 5.73 mg. of it were refluxed for 4 hours with 0.4 cc. of 6 *N* HCl at 100°. The hydrolysate was diluted to 1 cc. with water, the humin was filtered off and washed, and the filtrate and washing were freed from HCl by placing in a vacuum desiccator over solid caustic soda. The residue was dissolved in 0.30 cc. of H_2O and neutralized with 0.03 cc. of 0.2 *N* NaOH, and 0.08 cc. (corresponding to 1.43 mg. of the material) was sampled for the paper chromatography. Of the DNP-derivative, 6.32 mg. were taken and hydrolysis was effected with 0.45 cc. of 6 *N* HCl similar to above. The hydrolysate freed from humin was shaken with ether three times and after drying, taken up in 0.30 cc. of H_2O, followed by sampling 0.068 cc. (corresponding to 1.43 mg. of the material) of the solution. Figures obtained are listed in Table IA.

The other Partial Group Lipoid specimen (Prep. II) containing only one molecule of ethanolamine (It contained two acylglycerophosphoryl residues as Prep. I). 6.51 mg. were hydrolyzed with 0.45 cc. of 6 *N* HCl, and the hydrolysate, diluted and liberated from humin, was dried over NaOH, dissolved in 0.29 cc. of H_2O and neutralized with 0.03 cc. of 0.2 *N* NaOH. 0.08 cc. of the solution (corresponding to 1.62 mg. of the substance) was chromatographed. On the other hand, a part of the material was also changed into DNP-derivative by prolonged reaction with DNFB (60 hours in an ice chest) and 6.05 mg. of it were hydrolyzed with 0.40 cc. of 6 *N* HCl. The hydrolysate freed from humin were treated with ether as the corresponding hydrolysate above. And the residue

dried up was dissolved in 0.34 cc. of H_2O and neutralized with 0.03 cc. of 0.2 *N* NaOH, and a 0.10 cc. portion (corresponding to 1.62 mg. of the substance) was chromatographed. Table IB shows the analytical figures here obtained.

TABLE I

Analysis of the Ninhydrin-Positive Components in the Hydrolysate of the Partial Group Lipoid Specimens as such and after Treatment with DNFB

Component	Optical density after full ninhydrin reaction of spots		Amino-N (γ)		Approximate molecular ratios	
	Specimen as such	Specimen after tr. w. DNFB	Specimen as such	Specimen after tr. w. DNFB	Specimen as such	Specimen after tr. w. DNFB
A: 1.43 mg. each of Prep. I and its DNP-derivative analyzed						
Glutamic acid	0.348	0.325	6.5	6.2	2	2
Glycine	0.173	0.017	3.3	0.2	1	0
Serine	0.184	0.162	3.0	3.2	1	1
Chondrosamine	0.315	0.093	5.9	0.9	2	0
Ethanolamine	0.262	0.155	6.3	3.4	2	1
B: 1.62 mg. each of Prep. II and its DNP-derivative analyzed						
Glutamic acid	0.381	0.350	7.0	6.5	2	2
Glycine	0.182	0.025	3.5	0.3	1	0
Serine	0.205	0.244	3.7	4.1	1	1
Chondrosamine	0.296	0.042	5.6	0.4	2	0
Ethanolamine	0.145	0.025	3.5	0.2	1	0

Comments on the Amide Link of Ethanolamine

Judging from the findings above, the both Partial Group Lipoid preparations contained glycine and chondrosamine molecules in a state carrying a free NH_2-group. Of two molecules of ethanolamine in Prep. I, probably attached to different glycerophosphoryl rests, one possessed a free amino group and the other a bound. The latter is considered to make a bridge between one of the phosphatidyl rests and the polypeptide or carbohydrate moiety. However, the amino group of the sole ethanolamine molecule in Prep. II was found to have been attacked by DNFB, contrary to expectation. Since this specimen was submitted to the treatment with the agent for even 60 hours, the long exposure of it to alkali may have cleft the amide link between the NH_2-group of the ethanolamine and the probable terminal carboxyl of the polypeptide. To con-

firm this possibility, N-carbobenzoxyglutamyl-ethanolamine, N-phthalylglycyl-ethanolamine and N-carbobenzoxyglutamyl-glutamic acid were prepared and examined as to their stability against alkali and acid.

Preparation of the amides and peptide

According to Fourneau & Gonzalez[11], the amide is formed preferably to the ester, when ethanolamine is allowed to react with an equivalent amount of acylchloride, so that the hydroxyl of the amine was not protected in advance.

Carbobenzoxyglutamyl-ethanolamine. 0.3 g. of N-carbobenzoxyglutamic anhydride, prepared according to Bergmann & Zervas[12], was dissolved in 1 cc. of dry chloroform, 1 cc. of dry chloroform containing 0.01 cc. ethanolamine was added and the mixture was shaken violently. An oily mass came out with heat evolution and floated over chloroform. After standing for a while in ice water, it was carefully pipetted out and washed in another tube with portions of chloroform till nearly free from ethanolamine. The adhering chloroform was removed by vacuum distillation. The residual was dissolved in 0.5 cc. of ethanol, followed by addition of ethylacetate to slight turbidity, and the vessel wall was scratched under cooling. Crystals separated in small thin needles, which were filtered off. After recrystallization in a like manner, 180 mg. of a product melting at 116–118° were given. It was soluble in ethanol, methanol and water, but insoluble in chloroform, benzene and ethylacetate, and gave rise to no coloration on heating with the modified Stein & Moore's ninhydrin solution.

N (micro Kjeldahl) 8.71% (Calc. for $C_{15}H_{20}O_6N_2$ 8.64%).

Phthalylglycyl-ethanolamine. 0.3 g. of phthalylglycine (Billmann & Hartung[13]) was transformed into the acid-chloride with 0.4 cc. of $SOCl_2$[14] and dissolved in 10 cc. of abs. ether. On the other hand, 1 g. of ethanolamine hydrochloride was dissolved in 5 cc. of abs. methanol containing 5.86% sodium methylate and chilled well in an ice-salt mixture. NaCl formed was completely precipitated by the aid of a little volume of ether and filtered off, and the filtrate was dried with Na_2SO_4 and distilled to dryness in vacuo. The still-residue was taken up in 21 cc. of abs. ether, and to 3 cc. of this solution, under vigorous shaking, five 2 cc. portions of the phthalylglycylcholride solution and five 0.1 cc. portions of pyridine were added alternatively in the course of 15 minutes. The mixture was stood for 1 hour. The bulky white precipitate was centrifuged, dissolved in a little ethanol, and after cautious addition of ether to persistent turbidity, stood in an ice chest overnight. A deposit in fine needles was filtered off and dried. The product dissolved readily in ethanol and slightly in water and chloroform, but neither in ether nor in benzene. On heating, it contracted at 174° and turned into a brown liquid at 198°, and recrystallization did not change the behaviour. No coloration occurred, when it was boiled with the modified Stein & Moore's ninhydrin reagent.

N (micro Kjeldahl) 11.28% (Calc. for $C_{12}H_{12}O_4N_2$ 11.29%).

Carbobenzoxyglutamyl-glutamic acid. It was prepared after Bergmann & Zervas.[12]

Behaviour in slight alkalinity

1% watery solutions of N-carbobenzoxyglutamyl-ethanolamine (a), N-phthalylglycyl-ethanolamine (b) and N-carbobenzoxyglutamyl-glutamic acid (c) were prepared. From each of them, five 0.2 cc. portions were pipetted out into test tubes separately. Four of them were then added to with 0.2 cc. of 2 *N* $NaHCO_3$ each and a fifth with 0.2 cc. of water. The mixtures I's, II's, III's and IV's were stood at room temperature (8–12°) for 1, 2, 3 and 4 days respectively, followed by neutralization with 1 *N* HCl. V's were stood for 5 days. Thereupon, they were heated with 0.5 cc. of the modified Stein & Moore's ninhydrin solution for 10 minutes in boiling water.

The results are tabulated below.

Compound (1% solution)	No. of mixtures				
	I	II	III	IV	V
(a)	−	+	+	++	−
(b)	−	+	+	++	−
(c)	−	−	−	−	−

(−: almost negative, +: plainly positive, ++: markedly positive.)

The solutions (a) and (b) were also heated with the $NaHCO_3$ solution for a short time instead of standing at room temperature. After this treatment, they gave strong ninhydrin reaction.

Apart from the experiment above, 1 cc. of the solution of N-phthalylglycyl-ethanolamine (b) was stood for 2 days together with 1 cc. of the $NaHCO_3$ solution in air and shaken with 2 cc. of 1% DNFB in ethanol for 2 hours. The mixture was followingly diluted with 20 cc. of water, acidulated with 1 *N* HCl and extracted with ether. Paper chromatography of the ethereal extract showed a spot of DNP-ethanolamine.

Thus it is most plausibly assumed that Prep. II of the Partial Group Lipoid was cleft at the amide link during the treatment with DNFB.

Behaviour in slight acidity

1% watery solutions of those synthetical compounds were acidulated with an equal volume of 0.2 *N* acetic acid (pH of the mixtures 2–3), maintained at 50°C for 1 hour and neutralized with 0.2 *N* NaOH. The mixtures were then heated at 100° with the modified Stein & Moore's ninhydrin solution for 10 minutes. No color developed by any of them. Moreover, the solutions same as above were mixed with an equal volume of 1 *N* acetic acid and placed in a boiling water-bath for 1 minute. Test with ninhydrin of the solution neutralized resulted again in negative. Namely, the compounds are quite stable against mild acids.

The Presence of N-Glycoside Linkage in the Partial Group Lipoid

Test with $HgCl_2$ and Fehling and Benedict reagents

Under the conditions of Masamune *et al.*[15] N-glycosides do not reduce $HgCl_2$ at pH 7.5–8.0, while they reduce Fehling and Benedict reagents. The Partial Group Lipoid specimens behaved just like N-glycosides in this respect.

Behaviour of the Partial Group Lipoid towards ninhydrin

Tested on a paper as below, peptides, amino acids and primary amines show most intensive coloration with ninhydrin at pH 7.0, whether their solutions at different pH's are previously kept at 50°C for a while or no. N-Glycosides give stronger coloration at a pH range from 3.0 to 5.0 than at pH's 7.0 and more even as such, but the distinction increases when their solutions are heated in advance. It was found that the Partial Group Lipoid resembled N-glycosides, as will be exemplified.—5 mg. of Prep. II of the Partial Group Lipoid were dissolved in 0.5 cc. of H_2O and 0.1 cc. portions were mixed with 0.1 cc. of McIlvain's 0.1 *M* citrate-0.2 *M* phosphate buffer of pH 2.0, 3.0, 5.0, 7.0 or 8.0 or 0.1 *M* carbonate buffer of pH 9.0. A small square of filter paper was spotted with 0.01 cc. each of the buffer-Partial Group Lipoid mixtures in a line and dried in air. The remaining part of the mixtures were kept at 50°C for 5 hours as stoppered, and thereafter a 0.01 cc. portion of them were put on another line on that paper parallel to the former and dried. The paper was then sprayed with a 1% ninhydrin solution in ethanol and hung in a moistened dark chamber at 50°C. Fig. 2 (A–D) shows not only the result with the Partial Group Lipoid but also those with glycyl-glycineethylester-N-(N-acetyl)-glucosaminide, glycylglycineethylester and glycylglycine. In the cases of these three compounds 1 mg. of substance was treated as 5 mg. of the Partial Group Lipoid were. Of the buffer-Partial Group Lipoid mixtures without antecedent warming, that of pH 7.0 showed maximum coloration due to the pre-existent free amino groups in the Partial Group Lipoid, whereas, of the mixtures after warming, those buffered to pH's 3–5 gave the most intensive coloration, suggesting that an NH_2-group had been set free by scission of the N-glycoside bond. The same result was given with the group carbohydrate from pig stomach mucus prepared as by Yosizawa,[16] whose study in the present connection will be published in detail elsewhere.

Glycylglycineethylester-N-(N-acetyl)-glucosaminide was prepared as follows. 0.51 g. of glycylglycineethylester and 0.6 g. of N-acetylglucosamine were taken up in 15 cc. of abs. methanol, and after addition of a drop of glacial acetic acid as a catalyst, boiled gently for 3 hours. Some precipitate occurring on cooling

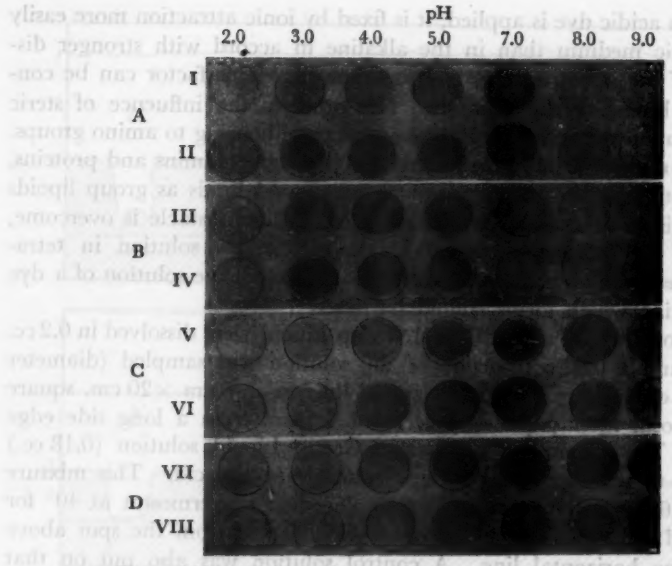

Fig. 2. Showing the ninhydrin spot test in various pH media. AI: the active fragment of the Group A human liver group lipoid (Prep. I) buffered to different pH's, AII: AI warmed at 50° for 5 hrs., BIII: glycylglycineethylester-N-(N-acetyl)-glucosaminide buffered to different pH's, BIV: BIII warmed as AII, CV: glycylglycineethylester buffered to different pH's, CVI: CV warmed as AII, DVII: glycylglycine buffered to different pH's, DVIII: DVII warmed as AII.

was filtered off, and the filtrate was concentrated in vacuo to a thick solution and precipitated by careful addition of ether. The deposit in fine needles was filtered, washed with ether and dried in vacuo over $CaCl_2$. Yield 0.8 g. It gave the positive indirect-Osaki-Turumi reaction, but not the direct, and melted at 188–190° under decomposition, having turned brown at 150°. It contained no crystal water.

N (micro Kjeldahl) 11.9% (Calc. for $C_{18}H_{25}O_8N_3$ 12.0%).

Retention analysis

That the Partial Group Lipoid contains a bound amino group which easily becomes free by warm mild acid has been established in a more precise manner by retention analysis.[17] To the end 0.05% azocarmine in water was employed at first. But the dye solution was replaced later with Wieland & Fischer reagent.[17] Two factors must be taken into consideration with respect to adaptation of the present analysis. One is the pH of the medium, because, if a negatively charged compound, for

instance, an acidic dye is applied, it is fixed by ionic attraction more easily in the acidic medium than in the alkaline in accord with stronger dissociation of the amino groups in the unknown. This factor can be controlled by buffering the solution. The other is the influence of steric hindrance upon the retention by the atoms neighbouring to amino groups. It must be remarkable in group carbohydrates, glucidamins and proteins, but can not be neglected even in such compound lipids as group lipoids that comprise a few amino acids and sugars. The obstacle is overcome, if Wieland & Fischer reagent a 0.1% cupric acetate solution in tetrahydrofurane containing 5% water is used in place of the solution of a dye as azocarmine which forms colloid particles.

5 mg. of Prep. II of the Partial Group Lipoid were dissolved in 0.2 cc. of water, and a 0.02 cc. portion of the solution was sampled (diameter of the spot circle 1 cm.) on a horizontal line on an 8 cm. × 20 cm. square of Toyo Roshi filter paper No. 2, ruled 1.5 cm. from a long side edge (bottom). The balance of the Partial Group Lipoid solution (0.18 cc.) was added to with an equal volume of 0.5 *N* acetic acid. This mixture (pH 2.2–2.6 against thymol blue) was placed in a thermostat at 40° for 1 hour and then 0.04 cc. was sampled 5 cm. apart from the spot above on the same horizontal line. A control solution was also put on that line at 5 cm. intervals with and without antecedent warm acetic acid-treatment. Acetic acid was evaporated off in vacuo as far as possible. The reagent of Wieland & Fischer was acidified with a few drops of glacial acetic acid until turbidity of the reagent disappeared, poured into a Petri dish to a height of about 0.5 cm. and after covering with a bell, placed in an incubator at 20°C. The paper spotted above was first held in the bell room to saturate with the vapor of tetrahydrofurane, and subsequently stood vertically in the solution. When the solvent front reached near the upper edge, the paper was removed from the dish, dried in air, and sprayed with 0.1% diphenylcarbazide in acetone. The area infiltrated with copper being colored greyish purple, gaps in it made by retention were noticed plainly. As is shown in Fig. 3, the gap owing to the Partial Group Lipoid, which had been warmed with the acetic acid, was obviously larger than that owing to the untreated. The same was the case with a synthetical N-glycoside and with the group carbohydrate, but not with edestin (The runs were started with 0.1, 5 and 5 mg. respectively of the substances).

Thus the experiments in the foregoing sections made clear the presence of an amide link and those above in this section that of an N-glycoside link.

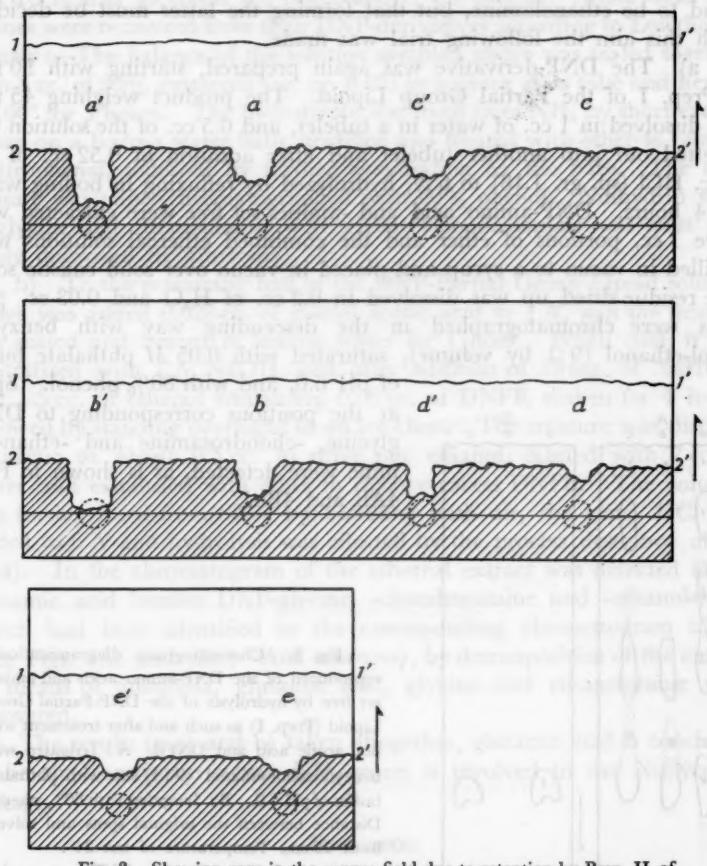

Fig. 3. Showing gaps in the copper field due to retention by Prep. II of the active fragment of Group A human liver group lipoid (a and a'), that of hog lung group lipoid (b and b'),[18] glycylglycineethylester-N-(N-acetyl)-glucosaminide (c and c'), group carbohydrate from hog stomach mucus (d and d') and edestin (e and e') as such (a–e) and after treatment with warm acetic acid (a'–e'). Irrigation at room temperature.
1–1': tetrahydrafurane front, 2–2': copper front.

Constitutent whose NH_2-Group can be set Free by Mild Acids

From above it is clear that there occur in the Partial Group Lipoid two kinds of unstable >N-C- union the amide- and N-glycoside-link. The constituent whose amino group partakes in the former linkage has been

found to be ethanolamine, but that forming the latter must be decided. With this aim the following trial was made.

a) The DNP-derivative was again prepared, starting with 50 mg. of Prep. I of the Partial Group Lipoid. The product weighing 45 mg. was dissolved in 1 cc. of water in a tubelet, and 0.5 cc. of the solution was pipetted out into another tubelet and after addition of 0.52 cc. of the conc. HCl (sp. gr. 1.18) to 6 *N*, hydrolyzed by refluxing in boiling water for 4 hours. DNP-amino acid and -amines set free were extracted with three 1 cc. portions of ether and the combined ethereal solutions were distilled in vacuo to a syrup and placed in vacuo over solid caustic soda. The residue dried up was dissolved in 0.2 cc. of H_2O and 0.03 cc. portions were chromatographed in the descending way with benzylalcohol-ethanol (9:1 by volume), saturated with 0.05 *M* phthalate buffer of pH 6.0, and with 80% phenol. Spots at the positions corresponding to DNP-glycine, -chondrosamine and -ethanolamine were detected, as is shown in Figs. 4Ab and 4Bb.

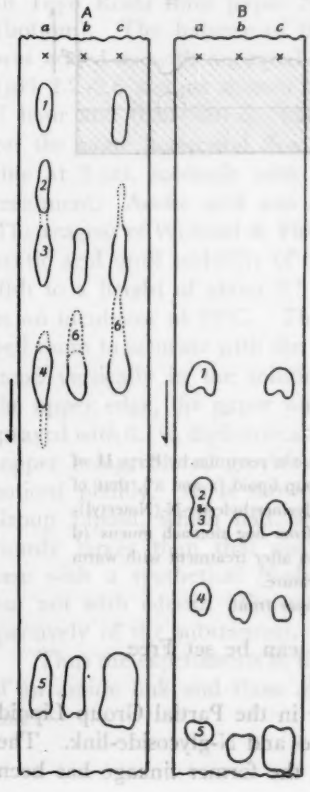

Fig. 4. Chromatograms diagrammatically reproduced of the DNP-amino acids and amines set free by hydrolysis of the DNP-Partial Group Lipoid (Prep. I) as such and after treatment with hot acetic acid and DNFB. A: Irrigated with benzylalcohol-ethanol (9:1) sat. with phthalate buffer of pH 6.0. B: Irrigated with 80% phenol. Distance between the original spots and solvent front 45 cm. Temperature of test 24°.

a: reference run with DNP-amino acids, -ethanolamine and -glucosamine, b: ether extract of the immediate hydrolysate of the DNP-Partial Group Lipoid, c: ether extract of the hydrolysate of the DNP-Partial Group Lipoid after treatment with hot acetic acid and DNFB. 1: DNP-glutamic acid, 2: DNP-serine, 3: DNP-glycine, 4: DNP-glucosamine (DNP-chondrosamine is located at the same position), 5: DNP-ethanolamine, 6: probable dinitrophenol.

Note that the DNP-ethanolamine spots in c's are enlarged due to overlapping each other of this component and dinitroaniline.

To further ascertain the result above, the original amino acid and amines were recovered from their DNP-derivatives according to Lowther,[19] namely:—The balance of the solution sampled above was sealed together with 3 cc. of 30% ammonia (sp. gr. 0.85) in a pyrex glass tube and heated at 100° for 3 hours. The hydrolysate was freed from NH_3 by short evaporation on a boiling water bath, acidified and treated with ether to remove dinitrophenol, followed by further evaporation on the bath. The condensate was paper-chromatographed with 80% phenol. Ninhydrin spots of glycine and ethanolamine were given (Chondrosamine was most probably destroyed by ammonia).

b) To the remaining half of the DNP-Partial Group Lipoid solution above was added 0.028 cc. of glacial acetic acid to 1 *N*, and the mixture was sealed and heated in boiling water for 1 hour. When cold, it was neutralized with 1 *N* NaOH, and after addition of 15 mg. of $NaHCO_3$ and 1.5 cc. of ethanol containing 0.03 cc. of DNFB, shaken for 4 hours, followed by standing overnight in an ice chest. The mixture was distilled in vacuo to about 0.5 cc. to drive out ethanol, diluted with 2 cc. of water and exhausted with ether to remove excess DNFB. The solution was further freed from ether by bubbling with air, the conc. HCl was added to 6 *N* and hydrolysis was effected by the process described above in a). In the chromatogram of the ethereal extract was detected DNP-glutamic acid besides DNP-glycine, -chondrosamine and -ethanolamine which had been identified in the corresponding chromatogram in a). (See Figs. 4Ac and 4Bc.) And moreover, by decomposition of the extract by means of ammonia, glutamic acid, glycine and ethanolamine were recovered.

The results in a) and b) taken altogether, glutamic acid is concluded to be the constituent whose NH_2-group is involved in the N-glycoside linkage.

Discussion

In the deductions above the writer tentatively assumed that, in the molecule of the Partial Group Lipoid, the ordinary lipid components, amino acids and sugars are grouped together into a phosphatidyl-ethanolamine, a polypeptide and a polysaccharide respectively. Under this postulate, which appears rational, discussions will be made. Of the amino acids glutamic acid, glycine and serine, glycine bears the amino group in a free state so that it must be combined through its carboxyl with serine-OH, forming a side chain of the polypeptide, because the terminal amino group of the polypeptide belonging to one of the glutamic acid molecules is bound with the carbohydrate moiety as mentioned. If then, the parent chain is made up of two molecules of glutamic acid and

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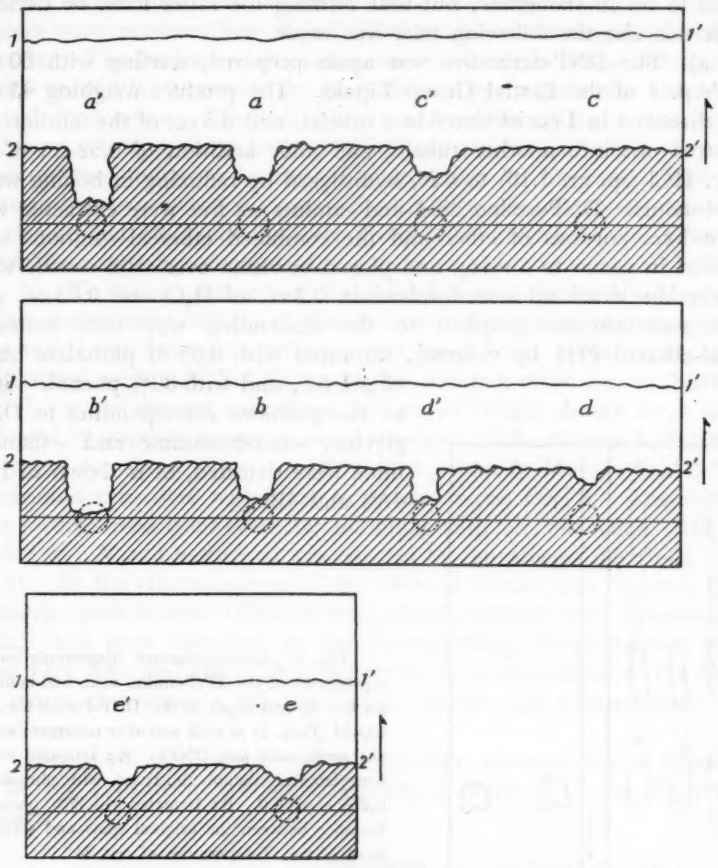

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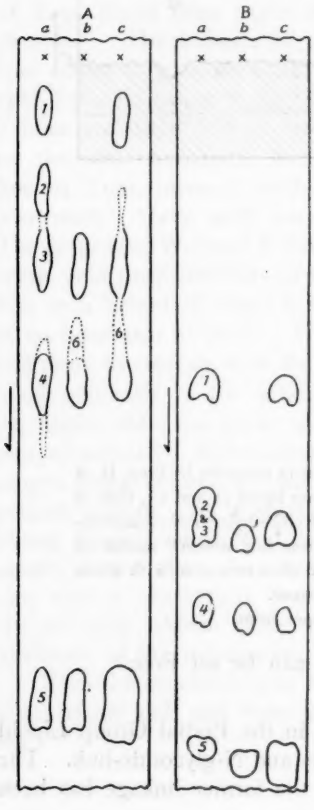

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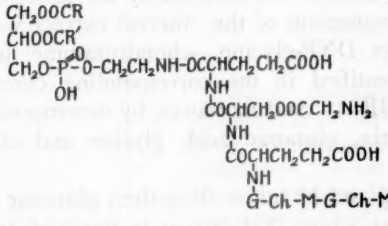
one molecule of serine, and the glutamic acid molecule other than the one above probably stands at the other terminal of the chain, since the occurrence of glutamyl-glutamyl residue is rather rare in proteins. Thus, one of the carboxyl groups, whether α- or γ-, of this glutamic acid molecule and the amino group of the ethanolamine molecule (in Prep. II) or one of the ethanolamine molecules (in Prep. I) are regarded as joined together.

With respect to the arrangement of monosaccharides (chondrosamine, galactose and mannose in molecular proportions of 2:3:2) in the polysaccharide chain, the sugar at the reducing end of the chain can not be chondrosamine. Because, despite that the chondrosamine molecules carry their NH_2-group free, Elson & Morgan test without acid hydrolysis of the Partial Group Lipoid, in which the polysaccharide is conjugated with the polypeptide N-glycosidically, gave rise to no positive reaction. Judging from their molecular ratios, the monosaccharides are most probably disposed either as G*-Ch-M-G-Ch-M-G or as G*-M-Ch-G-M-Ch-G (G=galactose, Ch=chondrosamine, M=mannose. * denotes the reducing hexose unit).

Taken altogether, the formula below expresses one of the most possible structures of Prep. II of the Partial Group Lipoid.



(*G=Galactose, Ch=Chondrosamine, M=Mannose*)

Summary

1. Preps. I and II of the Partial Group Lipoid (active fragment of Group A human liver group lipoid), which had been described formerly in an article[2] from this institute, were investigated regarding structure.

2. The sole ethanolamine molecule in Prep. II or one of the two ethanolamine molecules in Prep. I appears to be joined through its NH_2-group with a carboxyl belonging to the amino acid grouping in which glycine has been found to bear its NH_2-group in a free state.

3. The polysaccharide was found conjugated with the NH_2-group of one of the glutamyl rests in the polypeptide N-glycosidically.

4. Arrangement of the amino acids as well as that of the monosaccharides was discussed.

The expenses of this work were defrayed by a grant from the Education Department through the Grant Committee for Scientific Researches. H. Masamune.

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2) Masamune, Hakomori, Machara & Suzuki, *ibid.*, 1954, **59**, 231.
3) Wittcoff, The phosphatide, Reinhold Publ. Corp. New York, 1952, p. 93.
4) Hecht & Mink, Biochim. et biophys. acta, 1952, **8**, 641.
5) Sanger, Biochem. J., 1945, **30**, 507.
6) Kindly supplied by Mr. Satoru Masamune in the Chemical Department of the Faculty of Science.
7) Blackburn & Lowther, Biochem. J., 1951, **48**, 126.
8) Those solvents gave rise to tailing or heading of spots. The writer found later that 80% phenol and a phenol-butanol (1 : 3 by volume) mixture saturated with water give less diffuse spots, although they are not buffered.
9) Annison, James & Morgan, Biochem. J., 1951, **48**, 477.
10) Kindly given by Dr. Saburo Takizawa in the Applied Chemistry Department of the Faculty of Technology.
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14) Purified with quinoline and linseed oil (Fieser, Experiments in organic chemistry, 2nd ed., Reinhold Publ. Corp. New York, 1942, p. 381).
15) Masamune, Tsuiki & Ogawa, Tohoku J. Exp. Med., this volume, p. 157.
16) Yosizawa, *ibid.*, 1949, **51**, 51.
17) Wieland & Fischer, Cited in Kuwata's Chromatography (Jap.), Hirokawa Publ. Co. Tokyo, 1952, p. 163.
18) The writer expresses grätitude to Dr. Hidetake Hirata for the gift of a specimen (See Tohoku J. Exp. Med., 1954, **59**, 247).
19) Lowther, Nature, 1951, **167**, 767.

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On Proteins and Amino Acids

X. Determination of Amino Acids and Amines by Paper Partition Chromatography

By

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A method for the paper-chromatographic analysis of amino acids and amines was described in a foregoing article.[1] Since it requierd some skill, it has been improved since then. The procedure which is being now employed will be written in this account, although overlapping in several points with the former is not avoided.

Fischer *et al.*,[2] Block[3] and Brimley[4] tried to estimate amino acids in conformity to the alleged straight line relationship between the logarithms of their amounts and the areas colored with ninhydrin of their spots after irrigation. Bull *et al.*[5] and Polson[6] measured the transmissibility of light through the spots, fully developed with ninhydrin, of the amino acid chromatogram by means of a photocell and an amplifier. Giri and co-workers[7] extracted the spots, treated on the paper sliver similar to above, for spectrophotometric analysis, while Consden and others[8] and Pereira & Serra[9] first localized the spots by heating at 100°C with ultraviolet rays to followingly cut out and siphon out for the ninhydrin reaction, and Awapara & Landua[10] cut out the spots after incomplete colour development with ninhydrin on the paper sliver and effected the full reaction in test tubes. Contrary to many workers who submitted amino acid spots to maximum reaction with ninhydrin for the estimation, Hôzyô[11] determined the minimum threshold for ninhydrin reaction of the spots. He calculated the contents of amino acids in the unknown from the respective dilutions, where the amino acids are hardly detected after the reaction. On the other hand, Woiwod[12] and Schroeder *et al.*[13] changed the amino acids after extraction from the chromatogram into cupric salts by the aid of $Cu_3(PO_4)_2$. The former auther determined the combined copper absorptiometrically with sodium diethyl-dithiocarbamate and the latter group of investigators iodometrically. Further-

more, Wieland & Wirth[14] processed irrigation with cupric acetate-containing tetrahydrofurane to resort to retention analysis with rubeanic acid.

However, any of the methods is not adequate enough for precise quantitative analysis, particularly because of the difficulty in giving well-defined spots by irrigation. We carefully examined the conditions one by one and combined the methods into an elaborate for determination of not only amino acids but also amines including hexosamines. The improvements may be itemized as follows.

1) The whole manipulations to prepare the solution which is to be sampled on a paper are processed all in one tubelet to avoid the loss of substance in this course.

2) A solvent or solvents, which are changed basic on the buffer-impregnated paper, cause partial decomposition of hexosamines so that they are not employed for developing the chromatogram of this class of compounds.

3) If a solvent such as phenol is evaporated off by heating after irrigation, no small part of the material is often carried away together.[15] Hence a greater part of the solvent run is washed out with ether.

4) Such insufficiencies of irrigation as lengthening, overlapping, tailing and heading of spots entail significant error of analysis. But they can not be easily made good by controlling pH and quantity of the solution to be sampled, kind of solvent, temperature of test, quality of paper and so on. So clear-cut separation of aspartic and glutamic acid, of serine and glycine, of hexosamine and alanine, and of hexonebases between themselves has been long given up, being made still more difficult due to unavailableness of ammonia for the present aim. Nevertheless, Karnovsky & Johnson[16] recommended buffered solvents for separating penicillins from one another, Blackburn & Lowther[17] for obtaining the chromatogram of DNP-amino acids and McFarren[18] for minimizing the Rf change of amino acids. We substantiated the announcement of the authors and could obtain distinct spots of aspartic and glutamic acid, of serine and glycine and of hexosamine and alanine sharply separated from each other in respective pairs.

5) Quantitative estimation of the spots is carried out by following Awapara & Landua in the main, which is comparatively simple and reproducible, with modifications: i) Ninhydrin reagent for full colour development. Stein & Moore's ninhydrin reagent[19] was found preferable to those of Awapara & Landua and of Pereira & Serra in obtaining a stable and intensive colour, but was adapted after lowering the concentration of the expensive ninhydrin to 1% and replacing ethylcellosolve for methylcellosolve. Namely, 1 g. ninhydrin and 0.08 g.

$SnCl_2 \cdot 2H_2O$ is dissolved in a mixture of 50 cc. of 0.2 *M* citrate buffer of pH 5.0 and 50 cc. of ethylcellosolve.

ii) Vessel for the full ninhydrin reaction. A flat vessel of 8 cm. height and 3 cm. and 0.5 cm. sides is used in order to evenly heat the parts of the reaction mixture.

iii) Rinsing the vessel and paper fibres as well as dilution of the mixture after the reaction was processed with water by Awapara & Landua and with a phosphate buffer of pH 7.0 by Pereira & Serra. But these fluids allow quick decolorization of the solution, and particularly when the latter is diluted with water, the extinction of light decreases by more than 1/5 even within 20 minutes. Contrariwise, 75% ethanol containing 50–80 mg. of $CoSO_4 \cdot 7H_2O$ per *l.* proved to enormously stabilize the colour, maintaining constant for 24 hours.

iv) The solution after ninhydrin reaction and washings are transferred to a volumetric flask through a hardened filter paper with suction. Paper fibres must be completely removed, otherwise faulty analysis often ensues.

6) Reproducibility of the present method. By the technique here the analytical recoveries of amino acids and amines reach beyond 95%. Since, however, it is not perfectly reproducible, it is advisable to secure the determination in each series of analysis by standardizing the absorption by control runs, carried out in parallel with the main, with definite amounts of the amino acids and amines to be analyzed.

Procedure of Analysis

Preparation of a hydrolysate and sampling

An accurate amount (several milligrams) of a sample is weighed in a tubelet of 1 cm. diameter and 1.5 cm. height and refluxed with 6 *N* HCl in boiling water. When cold, the hydrolysate is placed in a vacuum desiccator over solid caustic soda to remove HCl as far as possible. The residue is taken up in a definite volume (0.1 cc. or so) of water, slightly alkalized to pH 7.5–8.0 with 0.2 *N* NaOH, whose volume employed is exactly measured, and after agitation with a small glass rod, centrifuged without freeing of the rod. On the other hand, a 6 cm. wide and 60 cm. long paper sliver (Toyo filter No. 2) is sprayed with the buffer solution same as that in the solvent to be used afterwards for irrigation, i.e., 0.05 *M* borate buffer of pH 7.5 or 0.05 *M* borax, (See below) and dried in air. An aliquot of the centrifuged supernatant is sampled on a half of a horizontal line ruled 10 cm. from the bottom of the sliver, and on the other half of the line is put exactly 0.01 cc.[20] of the standard solution containing 10γ each as NH_2-N in 0.03 cc.[21] of the same amino acids and

amines as are present in the unknown. On another sliver impregnated with the same buffer are pipetted 0.02 cc. and 0.03 cc. portions of the standard solution side by side in a similar manner. The two slivers are subjected to parallel descending irrigation.

TABLE I

Extinction Coefficients of Amino Acids and Amines after Ninhydrin Reaction

Name	Compound Wt. (γ) as amino-N	Extinction coefficients when analysis was processed	
		1) on individual compounds sampled separately	2) after irrigation of a mixture of compounds‡
Aspartic acid*	4	0.235	0.225 (95.7)
	8	0.435	0.425 (97.6)
	12	0.650	0.625 (96.2)
	14	0.732	0.725 (99.0)
Glutamic acid*	4	0.228	0.215 (95.1)
	8	0.438	0.425 (97.0)
	12	0.654	0.645 (98.6)
	14	0.775	0.760 (98.0)
Glycine*	4	0.215	0.205 (95.4)
	8	0.435	0.420 (96.5)
	12	0.645	0.630 (97.6)
	14	0.755	0.740 (98.0)
Serine*	4	0.245	0.240 (98.0)
	8	0.476	0.453 (95.2)
	12	0.655	0.640 (97.7)
	14	0.760	0.745 (97.4)
Glucosamine†,§	4	0.205	0.195 (95.0)
	8	0.380	0.375 (97.3)
	12	0.585	0.565 (96.5)
	14	0.762	0.755 (99.2)
Alanine*	4	0.220	0.215 (97.6)
	8	0.420	0.410 (97.6)
	12	0.615	0.601 (97.7)
	14	0.715	0.700 (97.8)
Ethanolamine*	4	0.245	0.233 (95.1)
	8	0.412	0.395 (95.8)
	12	0.568	0.565 (99.3)
	14	0.645	0.640 (99.2)

* Irrigated with Solv. I. † Irrigated with Solv. II. ‡ In parentheses are given the percentages of the respective recoveries of individual compounds sampled separately (1)). § Chondrosamine did not differ from glucosamine in the present respects.

Solvents and irrigation

A mixture of 0.05 *M* borate buffer of pH 7.5 and phenol (2:8) (Solv. I; pH 5.7) is applied, when hexosamine is analyzed, and a mixture of 0.05 *M* borax and phenol (2:8) (Solv. II; pH 6.6) for the analysis of amino acids and amines other than hexosamine. NH_3 is not used at all. Solvent front usually run 50 cm. Temperature of test 22°±1°C. After

irrigation, the chromatograms are freed from most of phenol by quick passing through dry ether in a rectangular dish three times and then dried at about 50°C over an oven for several hours.

Colour development

The chromatograms are sprayed with a 0.025% ninhydrin solution in water-sat. butanol (When Solv. II has been employed, color development occurs slowly). Spots indicated by heating are cut out and separately inserted into flat vessels described above after shearing into several pieces, followed by pipetting in of 1 cc. portions of the modified Stein & Moore ninhydrin reagent. The vessels are covered each with an aluminum foil, set in a stand and placed in an actively boiling water-bath for 20 minutes. Thereupon they are soon cooled in water. The solutions are filtered into 25 cc. volumetric flasks under suction. The cut pieces of paper are washed into the respective flasks and the flasks are filled up to the mark, employing the alcoholic cobaltous sulfate-solution throughout. The blank is conducted with a part of the chromatogram, unstained in the preliminary indication and possessing about the average area of the spots.

Spectrophotometry

Apparatus, Spekker photometer. Extinction at 570 mμ is measured against the blank in a 1 cm. thick cell (Sometimes a 2 cm. thick cell is used) within 24 hours. The amounts of amino acids are read from the curves constructed with the figures obtained on the standard solutions.

In Table I are embodied the mean figures in three series of analysis on standard amino acids and amines sampled separately and their mixture sampled and irrigated. In either case the extinction coefficients for various quantities of each amino acid or amine fall on a straight line.

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H. Masamune.

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20) It is recommended to make an ultramicro pipette by utilizing a thermometer whose bulb is broken.
21) The standard solution must be freshly prepared.

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On Existence in Tissue Cell of Tryptase as a Second Intracellular Proteinase

By

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The question whether or no an animal tissue cell possesses a second proteinase, tryptase (trypto-proteinase) besides catheptase (catho-proteinase) has not yet been decidedly answered. Early, Hedin[1] described spleen α-protease which should be identified to tryptase, but Waldschmidt-Leitz and Deutsch[2] refused to admit an existence on spleen of such tryptase. They attributed transient hydrolysis which appeared to occur at pH ca. 8 to the result of the combined actions of both catheptase and ereptase. Thereafter Hedin[3] reported further that α-protease was enriched in saline extract of acid precipitate of ox spleen, with its optimum pH-value at 8.8–8.9. Kleinmann and Stern[4] first agreed with the opinion of Waldschmidt-Leitz,[2] but Rona and Kleinman[5] were obliged to recognize two maxima on the pH-curve of spleen proteolysis. Even if spleen exhibits tryptase activity, it may be premature to conclude that spleen cel possesses its own tryptase, because spleen contains many blood cells, and the actions of the tryptase of the latter, either leucocytes or lymphocytes, should be considered, for the existence of tryptase in leucocytes or in lymphocytes[6] is well established.

On the other hand, Matsuo[7] concludes that "Entero-tryptase" (casein or gelatin hydrolysis at pH 7.8) exists in the intestinal juice or on the intestinal mucosa of dog, being highly activated in the presence of dialyzed albumin solution. Hino[8] reported the same findings of the intestinal mucosa of rabbit. Recently Hayakawa[9] reported on human gastric mucosa the existence of the same enzyme activity, and it can be separated, through acid treatment, from catheptase and ereptase activities as is shown in the report of Hayakawa and Kawasaki[10] more recently. Ito and Hayakawa[11] studied further on "Entero-tryptase" and found that it was distributed widely on the whole length of gastrointestinal mucosa of rabbit. Morever, Mori[12] reported the same findings as those of Matsuo[7] of human intestinal juice taken from patient with

jejunal fistula.

Now it seems for us doubtless that gastro-intestinal mucosa possesses tryptic activity which should, at least, be distinguished from pancreas trypsin.

In this paper observations are designed to search for tryptase on several tissues of rabbit.

Experimental

The method adopted is essentially the same as previously described.[7]

Dialyzed albumin solution: 2 per cent albumin solution was dialyzed through cellophane sack for 5 or 10 days against aqua destilata, its final volume increasing to almost twice of the initial volume.

Reaction mixture: 10 cc. 2%-substrate-buffer-solution+2 cc. enzyme solution (tissue homogenate 1 : glycerin water (1 :1) 3)+10 cc. dialyzed albumin solution or 10 cc. buffer solution. Control experiment in which either enzyme or substrate solution is substituted with buffer solution of equal quantity in the presence or absence of dialyzed albumin solution, was conducted under same condition.

Measurement of proteolytic activity: Increased acidity with respect to each 4 cc. of digestion mixture after incubation at 37°C under toluene, was formol-titrated with N/10-NaOH, and only corrected values are tabulated.

Results with Discussion

Gastric mucosa shows, besides catheptase, casein hydrolyzing activity even in slightly alkaline medium, but it is, after twenty four hours' digestion, so weak to be still questionable. The hydrolysis becomes apparenter first after relatively long while of digestion, and its optimal pH-value appears to be near 7.8 (Table I). Such activity can be always demonstrated on all animals examined, and hydrolysis amount of casein or gelatin is, even if it is not so large, fairly constant (Table II). And such activity is activated remarkably in the presence of dialyzed albumin solution, and hydrolysis of casein or gelatin becomes first doubtlessly apparent (Table III).

In the liver proteolysis the similar results are produced (Tables I & IV), but in this case the hydrolysis amount of gelatin appears somewhat smaller than that of casein (Tables I & IV). However, in the case of gastric mucosa proteolysis the former is nearly equal to or seems to exceed the latter somewhat (Table II). Albumin activation of this activity of the liver is, as well as that of gastric mucosa, also remarkable (Table V).

TABLE I

Casein Hydrolyzing Activity of Liver or Gastric Mucosa of Rabbit at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Enzyme	Time in hrs.	pH								
		3.5	4.0	4.5	5.0	7.0	7.4	7.8	8.2	8.6
Liver	24	0.1	0.15	0.15	0.05	0.15	0.2	0.2	0.1	0.1
	48	0.15	0.15	0.2	0.15	0.25	0.25	0.3	0.25	0.2
	72	0.15	0.2	0.3	0.25	0.25	0.3	0.35	0.25	0.25
Gastric mucosa	24	0.1	0.1	0.1	0.1	0.15	0.15	0.15	0.1	0.1
	48	0.1	0.15	0.15	0.15	0.2	0.2	0.2	0.2	0.15
	72	0.15	0.2	0.3	0.25	0.2	0.2	0.25	0.2	0.2

TABLE II

Proteolytic Activity of Rabbit Gastric Mucosa at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Substrate		Casein			Gelatin			Pepton		
Time in hrs.		24	72	120	24	72	120	24	72	120
No. of animals	2	0.15	0.3	—	—	—	—	—	—	—
	5	0.15	0.4	—	—	—	—	—	—	—
	8	—	—	—	0.1	0.45	—	—	—	—
	9	—	—	—	0.1	0.4	—	0.35	0.5	0.55
	11	0.1	0.4	0.5	0.1	0.45	0.5	0.4	0.55	0.55
	15	0.15	0.3	0.4	0.15	0.45	0.5	—	—	—
	16	0.2	0.35	0.4	0.15	0.45	0.5	—	—	—
	19	0.15	0.35	0.4	0.1	0.35	0.4	0.35	0.5	0.75
	20	0.15	0.4	0.45	—	0.3	—	0.4	0.5	0.75

TABLE III

Albumin Activation of Tryptase Activity of Rabbit Gastric Mucosa at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Substrate	Time in hrs.	Without albumin	With dialyzed ovalbumin
Casein	24	0.1	0.25
	72	0.35	0.8
	120	0.45	0.85
Gelatin	24	0.1	0.2
	72	0.45	0.8
	120	0.45	0.85
Without substrate (Control)	24		0.05
	72		0.25
	120		0.25

TABLE IV

Tryptase Activity of Rabbit Liver at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Substrate		Casein				Gelatin		
Time in hrs.		24	48	72	120	24	72	120
No. of animals	1	0.1	0.25	0.5	—	0.1	0.2	0.3
	2	0.15	0.2	0.3	—	—	—	—
	3	0.2	0.25	0.3	—	—	—	—
	4	0.2	0.25	0.3	—	—	—	—
	7	0.15	0.2	0.3	—	—	—	—
	8	0.2	0.2	0.2	—	—	—	—
	10	0.1	—	0.25	0.3	0.15	0.2	0.3
	11	0.15	—	0.25	0.4	0.1	0.2	0.3
	12	0.1	—	0.25	0.35	0.1	0.2	0.3
	13	0.1	—	0.25	0.3	0.1	0.15	0.15
	14	0.15	—	0.3	0.35	0.1	0.15	0.3

TABLE V

Albumin Activation of Rabbit Liver Tryptase at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Substrate	Time in hrs.	Without albumin	With dialyzed ovalbumin
Casein	24	0.2	0.3
	72	0.3	0.55
	120	0.4	0.75
Gelatin	24	0.15	0.2
	72	0.25	0.45
	120	0.35	0.65
Without substrate (Control)	24	—	0.1
	72	—	0.2
	120	—	0.25

These results are sufficiently similar to those obtained with human gastric mucosa[9-10] or consistent with the findings with "Enterotryptase"[7,11]. We are, therefore, disposed to admit an existence of tryptase also on the liver as well as on the gastric mucosa of rabbit. But these activities appear to be weaker than those of human gastric mucosa.

Besides tryptase, ereptase activity is shown on the liver or on the gastric mucosa (Tables VI & II). When enzymological comparison is made between gastric mucosa and liver, it is noted that tryptase activity is found stronger in the former and that ereptase is, on the contrary, so in the latter. These suggest that tryptase, existence of which is now the chief subject, is not identical with ereptase.

TABLE VI

Ereptase Activity of Rabbit Liver at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH. (substrate pepton)

Animal No.	Time in hrs.		
	24	72	120
15	0.65	0.8	0.95
16	0.55	0.7	1.1
17	0.55	0.75	1.1
18	0.55	0.65	1.0
19	0.7	0.75	0.8
20	0.65	0.85	0.85

Next, we carried out observations on kidney as before: Casein or gelatin is, already after twenty four hours' digestion, hydrolyzed markedly, and the amounts of hydrolysis increase during the lapse of the time and become, after seventy two hours, much higher (Table VII). This activity of kidney also appears fairly constant throughout all animals examined (Table VII), and we were surprised to find that tryptase on the kidney is very strong compared with that on the gastric mucosa or that on the liver. Therefore, its existence on kidney appears to be undoubted. Hydrolysis amount of casein in this case is found nearly equal to that of gelatin or slightly exceeding the latter. Ereptase activity of the kidney is found very strong also (Table VII).

TABLE VII

Ereptase and Tryptase Activities of Rabbit Kidney at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Substrate / Time in hrs. / Animal No.	Casein			Gelatin			Pepton	
	24	72	120	24	72	120	24	72
15	0.3	0.9	1.25	0.4	0.7	1.1	1.0	1.15
16	0.25	0.95	1.2	0.3	0.5	1.19	1.2	1.25
17	0.4	1.1	1.25	0.3	0.65	1.1	1.0	1.15
20	0.35	1.15	1.25	—	—	—	1.05	1.2
13	0.35	1.15	1.25	—	—	—	1.1	1.2
8	—	—	—	0.35	0.75	—	1.1	1.3
9	—	—	—	0.35	0.9	—	1.15	1.4

Tryptic hydrolysis of ovalbumin or of serum albumin by the liver, the gastric mucosa or by the kidney, is always negative (Table IX).

TABLE VIII

Hydrolysis of Serum Albumin or Ovalbumin at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.

Animal No.	Substrate	Time in hrs.	Enzyme solution		
			Kidney	Gastric mucosa	Liver
8	Ovalbumin (Takeda)	24	0	0	0.05
		48	0	0	0.05
		72	0.05	0.05	0.1
	Serum albumin (E. Merck)	24	0	0.05	0
		48	0	0.05	0
		72	0.05	0.05	0.1
9	Ovalbumin (Takeda)	24	0	0.05	0
		48	0	0.05	0
		72	0.05	0.05	0.1
	Serum albumin (E. Merck)	24	0	0.05	0.05
		48	0.05	0.05	0.05
		72	0.1	0.05	0.1

TABLE IX

Spleen Proteolysis at pH 7.8

Increases of acidity in 4 cc. digestion mixture; cc. of 0.1 *N* NaOH.
(substrate casein)

Time in hrs.	Animal No.	
	19	20
24	0.1	0.15
72	0.25	0.3
120	0.35	0.4

These proteins appear to be resistant to these enzyme actions.

Tryptase observed above appears to be characterized by delayed action, apparent hydrolysis being observed first after long digestion. In this respect, it may differ from pancreas trypsin, although it is still hard to determine exactly the origin of this ferment. But tryptase activity of kidney is, as shown above, very strong, and that of liver or gastric mucosa, through albumin activation, becomes more apparent. These facts suggest that it is not correct to ascribe their activities entirely to the ferment of leucocytes or lymphocytes. This view is supported by the fact that tryptase of spleen (Table X) which is thought to be richer in leucocytes or lymphocytes than kidney, liver or gastric mucosa is by no means stronger than that of kidney or exceeds those of liver and gastric mucosa. If lymphocytary or leucocytary origin of such ferment in each tissue should be universally advocated, catheptase in tissue too must be of lymphocytary

or leucocytary origin. There is no reason why tryptase only must be claimed to originate in the ferment of blood cells, though its activity in tissues other than kidney is, in its native state, not so strong. We are now of the opinion that tissue cells in which leucocyte or lymphocyte is implied, must contain a second proteinase, tryptase (tryptic proteinase, trypto-proteinase) in general.

Summary

1. Kidney, gastric mucosa or liver of rabbit has tryptase activity (hydrolysis of casein or gelatin) and its optimal reaction is near 7.8. Tryptic hydrolysis amounts are nearly constant with respect to each of these tissues throughout all the animals examined.

2. Tryptase activity of liver or gastric mucosa is activated in the presence of dialyzed albumin solution.

3. Tryptase activity of the gastric mucosa is found stronger than that of the liver, and ereptase activity (pepton, pH 7.8) is found stronger in the latter than in the former. Tryptase or ereptase activity of the kidney is, when compared with that of the gastric mucosa or those of the liver, exceedingly strong.

4. By liver proteolysis, the hydrolysis of gelatin appears smaller than that of casein, and by gastric mucosa or by kidney proteolysis, the former is nearly equal to the latter.

5. Hydrolysis of serum albumin or ovalbumin by these tissue homogenates at pH 7.8 is not recognizable.

6. The above results are fairly consistent with those obtained with human gastric mucosa, and acceptance of the general existence in tissue cell of tryptase as a second intra-cellular proteinase other than catheptic proteinase is advocated.

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In vitro Activation of Tissue Tryptase of Rabbit

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It was reported by Matsuo,[1] Hino[2] and Hayakawa[3] that the tryptase activity of the gastro-intestinal mucosa was highly activated in the presence of dialyzed ovalbumin or serum albumin solution. Previously, Hayakawa and Ito[4] suggested an existence in rabbit liver or kidney of such tryptase.

In this paper further observations are made on tissue tryptase which should be designated as a second intra-cellular proteinase.

Experimental

The method adopted is essentially the same as previously described.

Results

Activation of tryptase of gastric mucosa. (Table I) Hydrolysis of gelatin or casein at pH 7.8 is activated markedly in the presence of dialyzed ovalbumin or serum albumin solution. Serum albumin, which is, in itself, resistent to tryptase activity, also hydrolyzed to some extent. The activation becomes more apparent after seventy two-hours' digestion, and the hydrolysis amount of gelatin is almost equal to that of casein, whether activation is carried out or not. When pepton is used as the substrate, however, no activation is observed, and this accords with Hino's results.[2]

Activation of liver tryptase. (Table II) In the presence of dialyzed ovalbumin solution, hydrolysis at pH 7.8 of casein or gelatin is remarkably activated, and hydrolysis at pH 7.8 of pepton undergoes no activation. But it will be noted that amount of hydrolysis by liver of pepton is, similar to the previous observation, larger than that by gastric mucosa, i.e. ereptase is found stronger in the liver than in the latter either in its native or activated state.

Activation of kidney tryptase. (Table III) Casein or gelatin hydrolysis at pH 7.8 through kidney homogenate is marked enough in kidney tryptase to be unquestionable. Such strong tryptase is further activated in the

TABLE I

Albumin Activation of Tryptase of Rabbit Gastric Mucosa at pH 7.8

Increases of acidity in 4 cc. of digestion mixture: cc. of 0.1 *N* NaOH.

Substrate	Time in hrs.	Without albumin	With dialyzed	
			Ovalbumin	Serum albumin
Casein	24	0.05 ;0.1	0.25	0.35
	72	0.4 ;0.3	1.1	0.75
	120	0.5 ;0.4	1.15	0.85
Gelatin	24	0.05 ;0.1	0.2	0.2
	72	0.35 ;0.4	0.9	0.75
	120	0.45 ;0.45	0.9	0.85
Pepton	24	0.3 ;0.3	0.45	0.45
	72	0.55 ;0.5	0.85	0.65
	120	0.55 ;0.6	0.85	0.65
Serum albumin	24	0.05	0.2	—
	72	0.05	0.6	—
	120	0.1	0.6	—
Without substrate (Control)	24		0.05	0.1
	72		0.25	0.1
	120		0.25	0.1

TABLE II

Albumin Activation of Rabbit Liver Tryptase at pH 7.8

Increases of acidity in 4 cc. of the digestion mixture: cc. of 0.1 *N* NaOH.

Substrate	Time in hrs.	Without albumin	With dialyzed	
			Ovalbumin	Serum albumin
Casein	24	0.25 ;0.15	0.3	0.4
	72	0.3 ;0.25	0.55	0.7
	120	0.4 ;0.45	0.75	0.9
Gelatin	24	0.15 ;0.15	0.25	0.3
	72	0.25 ;0.3	0.4	0.5
	120	0.3 ;0.4	0.55	0.8
Pepton	24	0.85 ;0.75	0.85	0.8
	72	1.2 ;1.1	1.25	1.15
	120	1.35 ;1.2	1.35	1.25
Without substrate (Control)	24		0	0.05
	72		0.05	0.05
	120		0.05	0.1

same way, the hydrolysis amount becoming much greater. Ereptase of kidney, which is also found very strong, appears to undergo no activation.

Proteolytic activity of the other tissues. (Table IV) Casein hydrolysis at pH 7.8 through homogenate of heart muscle, testicle, mucosa of large intestine or spleen, is, after seventy two or one hundred and twenty hours, relatively apparent (Table IV). Such activity of each of these tissues

TABLE III

Albumin Activation of Rabbit Kidney Tryptase at pH 7.8

Increases of acidity in 4 cc. of the digestion mixture; cc. of 0.1 *N* NaOH.

Substrate	Time in hrs.	Without albumin	With dialyzed	
			ovalbumin	serum albumin
Casein	24	0.35 ;0.4	0.6	0.85
	72	1.15 ;1.2	0.65	1.5
	120	1.3 ;1.4	2.15	2.25
Gelatin	24	0.2 ;0.4	0.45	0.8
	72	0.65 ;0.85	1.4	1.1
	120	1.05 ;1.25	2.1	2.1
Pepton	24	1.4 ;1.15	1.2	1.2
	72	1.4 ;1.35	1.5	1.4
	120	1.4 ;1.4	1.5	1.55
Without substrate (Control)	24		0.2	0.2
	72		0.4	0.3
	120		0.5	0.4

TABLE IV

Proteolytic Activites of Various Tissues of Rabbit at pH 7.8

Increases of acidity* in 4 cc. of the digestion mixture; cc. of 0.1 *N* NaOH.

Tissue	Hydrolysis of											
	casein				gelatin				pepton			
	Number of cases	Time in hrs.			Number of cases	Time in hrs.			Number of cases	Time in hrs.		
		24	72	120		24	72	120		24	72	120
Brain	(7)	0.1	0.15	0.25	(1)	0.05	0.1	0.2				
Striped muscle	(5)	0.1	0.2	0.25	(6)	0.05	0.1	0.15	(5)	0.25	0.55	0.6
Heart muscle	(1)	0.15	0.3	0.4								
Testicle	(1)	0.15	0.25	0.55								
Blind intestine	(5)	0.1	0.2	0.25	(4)	0.05	0.1	0.15	(4)	0.4	0.6	0.7
Large intestine	(11)	0.1	0.2	0.4	(6)	0.05	0.1	0.25	(7)	0.55	0.65	0.7
Skin	(6)	0.1	0.15	0.25	(4)	0.1	0.1	0.15	(4)	0.15	0.2	0.2
Uterus	(2)	0.05	0.1	0.3	(2)	0.1	0.1	0.2	(3)	0.3	0.45	0.5
Spleen	(2)	0.15	0.3	0.4								

* Average values are shown.

except spleen, is highly activated in the presence of dialyzed albumin solution as before (Table V) and should, therefore, be attributed to an action of an enzyme which will be identified to tryptase found in liver, gastric mucosa or in kidney. Casein hydrolysis at pH 7.8 through brain,

mucosa of blind intestine, skin, uterus, or striped muscle is so small as to appear to lie within the limits of experimental error, and gelatin hydrolysis through each of these tissues is less marked (Table IV). From these the existence of tryptase in these tissues is still doubtful, but even in such cases the hydrolysis amount of casein or gelatin increases markedly in the presence of dialyzed albumin solution (Tables V & VI). Of these tissues too, therefore, it should be concluded that they are characterized by tryptase activity. Tryptase activities of these tissues are, therefore, relatively low in their native states and some of them appear to disclose their activities only after the activation.

It is noted however, that each of the tryptase of these tissues is, in

TABLE V

Albumin Activation of Rabbit Tissue Tryptase

Increases of acidity in 4 cc. of the digestion mixture; cc. of 0.1 *N* NaOH; pH of digestion 7.8; substrate, casein.

Tissue	Time in hrs.	Without albumin	With dialyzed* serum albumin
Heart muscle	24	0.15	0.3 (0.05)
	72	0.3	0.7 (0.1)
	120	0.4	0.95 (0.15)
Testicle	24	0.15	0.3 (0.05)
	72	0.25	0.6 (0.2)
	120	0.55	1.3 (0.2)
Blind intestine	24	0.1	0.2 (0.05)
	72	0.2	0.5 (0.05)
	120	0.3	0.7 (0.1)
Large intestine	24	0.1	0.3 (0)
	72	0.25	0.6 (0.05)
	120	0.35	0.8 (0.05)

* The values in parentheses are those obtained in the blank experiment without casein.

TABLE VI

a. Albumin Activation of Tryptase of Rabbit Striped Muscle at pH 7.8

Increases of acidity in 4 cc. of the digestion mixture: cc. of 0.1 *N* NaOH.

Substrate	Time in hrs.	Without albumin	With dialyzed serum albumin
Casein	24	0.1 ;0.1	0.25 ;0.25
	72	0.15 ;0.2	0.45 ;0.45
	120	0.3 ;0.3	0.85 ;0.65
Gelatin	24	0.1 ;0.05	0.25 ;0.15
	72	0.1 ;0.15	0.45 ;0.25
	120	0.15 ;0.2	0.7 ;0.45
Without substrate (Control)	24		(0.05 ;0.05)
	72		(0.15 ;0.05)
	120		(0.2 ;0.1)

b. Albumin Activation of Tryptase of Rabbit Brain or Skin at pH 7.8

Increases of acidity in 4 cc. of the digestion mixture: cc. of 0.1 *N* NaOH.

Substrate	Time in hrs.	Brain tryptase		Skin tryptase		
		Without albumin	With dialyzed serum albumin	Without albumin	With dialyzed ovalbumin	With dialyzed serum albumin
Casein	24	0.2	0.4	0.1 ;0.1	0.25	0.25
	72	0.35	0.8	0.15 ;0.15	0.35	0.35
	120	0.4	0.8	0.2 :0.2	0.55	0.6
Gelatin	24	0.1	0.4	0.05	0.15	
	72	0.25	0.55	0.1	0.4	
	120	0.25	0.55	0.15	0.45	
Without substrate (Control)	24		(0.1)		(0.05)	(0)
	72		(0.15)		(0.05)	(0.05)
	120		(0.15)		(0.1)	(0.1)

TABLE VII

Activating Ability of Dialyzed Albumin Solution after Dialysis against $FeSO_4$(M/100)

Increases of acidity in 4 cc. of the digestion mixture; cc. of 0.1 *N* NaOH; pH of digestion 7.8; substrate, casein.

Tissue	Time in hrs.	Without albumin	With dialyzed albumin	With Fe-albumin
Liver	24	0.1 ;0.15	0.4	0.15
	72	0.2 ;0.2	0.7	0.2
	120	0.35 ;0.3	0.95	0.3
Kidney	24	0.5 ;0.55	0.85	0.55
	72	1.05 ;0.95	1.55	0.95
	120	1.2 ;1.2	2.05	1.2
Striped muscle	24	0.05	0.2	0.05
	72	0.15	0.25	0.1
	120	0.25	0.45	0.15

the presence of dialyzed ovalbumin or serum albumin solution, activated in similar manner, and that degree of activation appears nearly equal regardless of kind of tissue, almost two times increase in hydrolysis amount being observed. And it is interesting that the dialyzed albumin solution loses its activating ability after it attained equilibrium with dialysis against fero-sulfate (m/100) solution (Table VII). This may afford some clue to the nature of the activation, the whole matter of which is still obscure. At any rate the results obtained give further suport to the hypothesis of general existence of tryptase in tissue.

With regard to ereptase activity, that of mucosa of blind intestine or large intestine, or uterus is relatively apparent (Table IV).

SUMMARY

1. Tryptase of gastric mucosa, liver or kidney of rabbit is activated in the presence of dialyzed ovalbumin or serum albumin solution in similar manner, while ereptase of these tissues undergoes no activation.

2. Tryptase of testicle, large intestine, or heart muscle is relatively apparent and undergoes albumin activation as well as above, but that of blind intestine, skin, brain, or striped muscle becomes first manifest after the activation with dialyzed albumin solution.

3. The degree of activation is almost constant throughout all experiments regardless of kind of tissue, and almost two times increase in hydrolysis amount is observed. The activating ability of dialyzed albumin solution disappears after it has attained the equilibrium for dialysis against $FeSO_4$ solution.

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Studies on Tissue Protease

I. Separation of Tryptase Activity from Catheptase or Ereptase Activity on Rabbit Liver Homogenates

(Directed by Asst. Prof. M. Hayakawa)

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The existence on tissue of tryptic proteinase has scarcely been paid any attention. Hayakawa and Ito[1] demonstrated that tryptase activities of rabbit tissues were activated or disclosed themselves in the presence of dialyzed albumin solution. Thereby it was suggested that rabbit tissues contained generally second proteinase tryptase proper to each tissue cell. But their activities are, in native state, not strong enough to be without question. Waldschmidt-Leitz and Deutsch[2] considered that transient or only weakly observable hydrolysis at alkaline medium of protein should be the results from the combined actions of catheptase and ereptase.

In this paper attempts to separate the activity of tryptase from those of the other enzymes were made following the acid and alkaline method of Utzino and Sakai.[3]

Experimental

Method

The method adopted are essentially the same as previously described.[1] *Acid or alkaline treatment for tissue homogenates:* Enzyme solution, any pH value desired for the treatment being brought forth by adding with 0.1 *N* NaOH or HCl solution, is incubated at 37°C for various periods and then neutralized in the same way, its volume being kept constant with H_2O. Another enzyme solution the volume of which is kept constant with water, is used for the control in the above test.

Corrected values with those of general controls (without substrate or enzyme solution) are tabulated, each denoting increased acidity with respect to each 4 cc. of digestion mixture in cc. of 0.1 *N* NaOH (Formol titration method).

Digestion mixture consisted of 5 cc. of enzyme solution as above treated and 20 cc. of substrate-buffer solution (*M*/15-phosphate buffer of pH 7.8 for tryptase or ereptase, *M*/10-citrate-HCl buffer solution of pH 4.5 for catheptase and of 1 cc. of buffer solution).

Cystein activation for catheptase: 1 cc. of 0.05 m-cystein-HCl (neutralized to pH 4.6 with 0.1 *N* NaOH) is mixed with 5 cc. enzyme solution and incubated at 37°C for thirty minutes.

Results with Discussion

1. Enzyme solution which does not contain catheptase (Tables I & II).

TABLE I

Tryptase Activity of Rabbit Liver Homogenate separated from Catho-Proteinase Activity by Means of Alkaline Treatment (1)

Alkaline treatment		Hydrolysis of casein											
		pH 7.8						pH 4.5					
		(A)*			(B)†			(A)*			(B)†		
time in min.	pH	time in hrs.			time in hrs.			time in hrs.			time in hrs.		
		24	72	120	24	72	120	24	72	120	24	72	120
	7.4	0.1	0.2	0.25	—	—	—	0.1	0.2	0.2	0.2	0.2	0.25
	7.8	0.1	0.1	0.15	—	—	—	0.15	0.15	0.15	0.35	0.35	0.35
60	8.2	0.05	0.1	0.15	—	—	—	0.1	0.15	0.2	0.2	0.2	0.25
	8.6	0.05	0.05	0.1	—	—	—	0	0	0	0	0	0
	9.0	0	0	0	—	—	—	0	0	0	0	0	0
	8.2	0.2	0.2	0.35	0.15	0.15	0.2	0	0	0	0	0	0
40	8.4	0.2	0.2	0.3	0.15	0.2	0.2	0	0	0	0	0	0
	8.6	0.2	0.3	0.3	0.15	0.2	0.25	0	0	0	0	0	0
	8.2	0.1	0.2	0.3	0.05	0.1	0.15	0.05	0.05	0.05	0	0	0
30	8.4	0.1	0.2	0.25	0.05	0.1	0.15	0.05	0.05	0.05	0	0	0
	8.6	0.1	0.15	0.2	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
	8.2	0.15	0.2	0.25	0.05	0.1	0.15	0.05	0.05	0.05	0.05	0.1	0.15
20	8.4	0.15	0.2	0.25	0.05	0.05	0.1	0.05	0.05	0.05	0	0.1	0.15
	8.6	0.15	0.15	0.2	0.1	0.1	0.15	0	0.05	0.05	0.05	0.1	0.15
Control without treatment		0.05	0.1	0.3	—	—	—	0.25	0.35	0.4	0.4	0.6	0.9
		0.2	0.3	0.35	—	—	—	0.2	0.3	0.4	0.45	0.55	0.8
		0.15	0.25	0.35	—	—	—	0.2	0.3	0.4	0.4	0.6	0.8

* without cystein; † activated with cystein.

TABLE II

Tryptase Activity Separated from Catho-proteinase Activity (2)

(substrate, gelatin; alkaline treatment: pH 8.4, 40 min.)

Alkaline treatment	time in hrs.	pH 7.8		pH 4.5	
		(A)*	(B)†	(A)*	(B)†
(+)	24	0.1	0.1	0	0
	72	0.15	0.15	0	0
	120	0.3	0.2	0	0
(−)	24	0.1	—	0.2	0.5
	72	0.2	—	0.3	0.6
	120	0.3	—	0.4	0.65

* without cystein; † activated with cystein.

Alkaline treatment is carried out at various pH values with variation for time of incubation. Sixty minutes treatment at pH 8.6–9.0 exerts disappearance both of catho-proteinase (casein, pH 4.5, with cystein activation) and of tryptase. Thirty or forty minutes treatment even at pH 8.2–8.6 preserves tryptase well, while catheptase perfectly disappears. Twenty minutes treatment at pH 8.2–8.6 or sixty minutes treatment at pH 7.4–8.2 is, however, unsatisfactory. Similar experiments with gelatin as substratum produce same results (Table II). So it is now evident that the alkaline treatment at pH 8.2–8.6 for thirty to forty minutes long is the most advantageous for excluding catheptase activity and preserving at the same time tryptase activity.

Catheptase is, as generally known, activated by cystein, but tryptase appears, contrary to that, to be rather inhibited by it. This was also the case when proteolysis of human gastric mucosa was studied by Hayakawa.[4] Hydrolysis at pH 4.5 of pepton is also activated by cystein (Table III), and the author prefers to admit on rabbit liver, as Utzino and Sakai[5] on swine liver, or Hayakawa[6] on human gastric mucosa did, an existence of catheptic pepton hydrolyzing enzyme. Similar treatment (pH 8.4–9.0, 40 min.) also results in disappearance of this enzyme, ereptase being preserved well (Table III).

It is now, at any rate, obvious that one can obtain, by means of the alkaline treatment presented above, an enzyme solution which contains tryptase and ereptase on one hand and does not contain catho-proteinase and catho-peptonase on the other.

2. Enzyme solution which contains tryptase and does not contain ereptase and catheptase.

To separate tryptase from ereptase, acid treatments on various modifi-

TABLE III

Ereptase Activity of Rabbit Liver Homogenate Separated from Catho-peptonase Activity by Means of Alkaline Treatment

Acid treatment		Hydrolysis of pepton											
		pH 7.8						pH 4.5					
time in min.	pH	(A)* time in hrs.			(B)† time in hrs.			(A)* time in hrs.			(B)† time in hrs.		
		24	72	120	24	72	120	24	72	120	24	72	120
40	8.2	0.6	0.85	1.1	0.75	1.05	1.2	0	0.1	0.1	0.05	0.05	0.05
	8.4	0.55	0.85	0.9	0.6	1.05	1.15	0	0	0.05	0	0.05	0.05
	8.6	0.5	0.8	0.85	0.7	1.1	1.15	0.05	0.05	0.05	0	0	0
	9.0	0.5	0.8	0.9	0.6	1.1	1.15	0	0	0	0	0	0
without treatment		0.6	0.8	0.85	—	—	—	0.35	0.45	0.6	0.55	0.6	0.85

* without cystein; † activated with cystein.

TABLE IV

Tryptase Activity of Rabbit Liver Homogenate Separated from Catho-peptonase Activity by Means of Acid Treatment

Acid treatment		Hydoolysis at pH 7.8 of					
		casein			pepton		
time in min.	pH	time in hrs.			time in hrs.		
		24	72	120	24	72	120
40	3.0	0.15	0.2	0.25	0	0	0
	3.5	0.2	0.25	0.3	0	0	0
	4.0	0.2	0.25	0.3	0	0	0
	4.5	0.25	0.3	0.35	0.1	0.2	0.3
	5.0	0.2	0.2	0.3	0.25	0.25	0.45
30	3.5	0.15	0.2	0.2	0	0	0
	4.0	0.25	0.35	0.4	0.05	0.1	0.1
	4.5	0.3	0.5	0.55	0.1	0.25	0.25
20	3.5	0.05	0.2	0.2	0	0	0
	4.0	0.3	0.3	0.35	0.05	0.05	0.1
	4.5	0.05	0.25	0.3	0.15	0.15	0.2
without treatment		0.2	0.25	0.35	0.7	0.75	0.9
		0.1	0.2	0.25	0.5	0.7	0.85
		0.15	0.2	0.3	0.6	0.65	0.8

cations are employed (Table IV), and the results are satisfactory, when enzyme solution is incubated at pH 4.0 for forty minutes, at pH 3.5 for

twenty or thirty minutes. Thirty minutes treatment at pH 5.0 is unsatisfactory. Treatment at pH 3.5 for thirty or forty minutes results in the complete disappearance of catho-proteinase and catho-peptonase simultaneously (Table V), and tryptase only is well preserved (Table IV). Such enzyme solution should, therefore, be said to contain only tryptase in it.

TABLE V

Disappearance of Catheptase Activity of Rabbit Liver Homogenate after Acid Treatment

Acid treatment		Hydrolysis at pH 4.5 of											
		casein						pepton					
		(A)*			(B)†			(A)*			(B)†		
time in min.	pH	time in hrs.			time in hrs.			time in hrs.			time in hrs.		
		24	72	120	24	72	120	24	72	120	25	72	120
40	3.5	0	0	0	0	0	0	0	0	0	0	0	0
	4.0	0.05	0.1	0.1	0.25	0.35	0.35	0	0	0	0	0.05	0.1
	4.5	0.1	0.2	0.2	0.35	0.35	0.35	0.15	0.15	0.2	0.15	0.25	0.25
30	3.0	0	0	0	0	0	0	0	0	0	—	—	—
	3.5	0	0	0	—	—	—	0	0	0	—	—	—
	4.0	0.05	0.1	0.15	—	—	—	0.05	0.1	0.15	—	—	—
	4.5	0.15	0.2	0.25	—	—	—	0.05	0.1	0.3	—	—	—
	5.0	0.15	0.15	0.25	—	—	—	0.2	0.3	0.35	—	—	—
without treatment		0.15	0.25	0.35	0.4	0.45	0.7	0.3	0.5	0.6	0.5	0.7	0.8
		0.2	0.25	0.35	—	—	—	0.35	0.45	0.55	—	—	—

* without cystein; † activated with cystein.

3. Tryptase after the acid treatment (Table VI).

Tryptase activity is, even after the acid treatment at pH 3.5, scarcely reduced (Table IV) and after treatment at pH 4.0–4.5 for over twenty minutes the amount of hydrolysis of casein appears to become higher than ever. No decrease in tryptic activity is observed even after treatment at pH 4 for one hour. As it is known that catheptase requires from 4.5 to 5.0 for its optimal pH range, such increase in tryptase activity may be explained as resulting from the autolytic action of catheptic enzymes.

4. Enzyme solution which contains ereptase but does not contain tryptase or catheptase (Tables VII & VIII).

Tryptase activity, as is evidenced already by the results in Table I, undergoes some inactivation through alkaline treatment. So resistance to alkaline of tryptase is further investigated, and the results show that tryptase disappears completely after incubation at pH 9.0 for twenty to

TABLE VI

Tryptase Activity of Rabbit Liver Homogenate after Acid Treatment

(Substrate, casein; pH 7.8)

Acid treatment		Hydrolysis of casein		
		time in hrs.		
time in min.	pH	24	72	120
60	4.0	0.15	0.35	0.45
	4.5	0.15	0.3	0.55
40	4.0	0.15	0.3	0.55
	4.5	0.3	0.4	0.55
30	4.0	0.2	0.3	0.45
	4.5	0.3	0.45	0.5
20	4.0	0.2	0.3	0.4
	4.5	0.3	0.35	0.5
10	4.0	0.1	0.25	0.35
	4.5	0.1	0.25	0.4
without treatment		0.1	0.2	0.35
		0.1	0.2	0.35
		0.05	0.25	0.35
		0.05	0.22	0.35
		0.05	0.25	0.35

TABLE VII

Resistance to Alkaline of Tryptase Activity of Rabbit Liver Homogenate

Alkaline treatment		Hydrolysis at pH 7.8 of					
		casein			gelatin		
		time in hrs.			time in hrs.		
time in min.	pH	24	72	120	24	72	120
10	8.6	0.05	0.1	0.2	0.05	0.05	0.15
	9.0	0	0.05	0.15	0	0.05	0.1
20	8.6	0	0.05	0.1	—	—	—
	9.0	0	0.05	0.05	0	0	0
30	8.6	0.05	0.1	0.2	0.05	0.1	0.15
	9.0	0	0	0	0	0	0
40	8.6	0	0.05	0.1	0.05	0.05	0.1
	9.0	0	0	0	0	0	0
without treatment		0.1	0.15	0.3	0.1	0.15	0.25

forty minutes (Table VII). Resistance to alkaline of ereptase is relatively strong (Table III) and so it is easy to obtain, through strong alkaline

treatment (pH 9.0, 60 min.), an enzyme solution containing only ereptase but not tryptase (Table VIII). Catheptase activity should, as obvious from the results in Table I, be missed in such enzyme solution.

TABLE VIII

Ereptase Activity of Rabbit Liver Homogenate Separated from Tryptase Activity after Alkaline Treatment

Alkaline treatment		Hydrolysis at pH 7.8 of					
		casein			pepton		
time in min.	pH	time in hrs.			time in hrs.		
		24	72	120	24	72	120
60	8.6	0.05	0.2	0.20	0.35	0.5	0.6
	9.0	0	0	0	0.3	0.4	0.5
without treatment		0.1	0.3	0.35	0.55	0.75	1.0

It is now possible to make us, for separating each enzyme activity from the others, of differences between resistance to alkaline or acid. Tryptase of rabbit liver is separated in such a way and can be observed independently of catheptase or ereptase. Therefore, hydrolysis at pH 7.8 of casein or gelatin can, even though its amount is not so high in native state, not be attributed simply to the combined actions of both catheptase and ereptase as was done by Waldschmidt-Leitz and Deutsch.[2)]

Existence of tryptase in rabbit liver is thus evidenced, and support for advocating its general existence in animal tissues is gained.

Summary

Rabbit liver proteolysis was studied, yielding the following results:

1. Enzyme solution which is incubated at pH 8.2–8.6 at 37°C for thirty or forty minutes exhibits tryptase activity (casein, gelatin, pH 7.8) and ereptase activity (pepton, pH 7.8) but does not exhibit catheptic proteinase and catheptic peptonase activity (casein, gelatin or pepton, pH 4.5, with cystein activation).
2. Tryptase is rather inhibited by cystein but ereptase is not.
3. Enzyme solution which is incubated at pH 3.5 at 37°C for forty minutes exhibits only tryptase activity, and neither ereptase nor catheptase activity can be observed on it.
4. Tryptase activity appears to increase slightly after incubation at pH 4.0–4.5 at 37°C.
5. Enzyme solution which is incubated at pH 9.0 at 37°C for sixty

minutes exhibits ereptase activity, and neither catheptase nor tryptase activity can be observed on it.

6. Existence on rabbit liver of tryptase is definitely evidenced.

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Comparison of Nucleic Acid Contents of Streptomycin-resistant and-dependent and Chloramphenicol-resistant Strain induced from *E. coli*.*

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During the growing process of bacteria, the increase and decrease in the amounts of nucleic acids, especially that of RNA is observed and the increase is conspicuous at the beginning of the incubation. This fact has been proved already chemically and histochemically by Malmgren *et al.*[1] Fish *et al.*[2] Boivin,[3] Belozersky[4] and other investigators.

In previous publication,[5] the author reported the nucleic acid contents of *S. abortus-equi* at the various culture stages and ascertained that RNA contents increased at the early stage of incubation, whereas DNA amounts are maintained almost constantly throughout all the culture stages.

From the above facts and Caspersson's theories that the metabolism of RNA is accompanied by protein synthesis, it can be inferred that in the early stage of incubation the synthesis of the bacterial protein may progress rapidly.

As mentioned above, the metabolism of nucleic acids and the synthesis of protein are indispensable for the growth of bacteria. On the other hand, antibiotics, which are thought to act bacteriostatically and bactericidally against some species of bacteria, are regarded to have a close relationship with the metabolism of nucleic acids, as indicated by Goldstein.[6] Among the antibiotics the author used streptomycin and chloramphenicol which show antibacterial activities mainly against Gram negative organisms, and isolated both the resistant and dependent strains from sensitive one of *E. coli* by the exposure to these antibiotics; after which the observations were made on the mutual relationship between these strains and the parent strain concerning the nucleic acid contents

* Read at the 25th General meeting of the Society of Japanese Bacteriologists on 7th April, 1952.

—as an indirect method to reveal the mode of actions of these antibiotics. That is, the author presumed that he would be able to get a general outline of the mechanisms of antibacterial actions and the manner in which a resistancy and dependency are acquired.

The present paper deals chiefly with (1) the determination of the comparative nucleic acid contents in the bacterial bodies of the streptomycin resistant,—dependent, chloramphenicol resistant and the parent strains at various culture stages, (2) the identification of free amino acids within the bacterial bodies of the streptomycin resistant,—dependent and the parent strains by paperchromatographic analysis and (3) the determination of the growth rates of each strain by the colony counting method.

Experimental

Streptomycin resistant,—dependent and chloramphenicol resistant strains were isolated from sensitive strain of *E. coli* (Umesawa) preserved in author's laboratory.

Antibiotics used: streptomycin trihydrochloride calcium chloride complex (National Institute of Health of Japan) which contained 500 mcg. of streptomycin base per mg.

Chloramphenicol (chloromycetin, Park-Davis Co.,) was equivalent to 950 mcg. per mg.

The above antibiotics were dissolved in water to adequate concentration and was sterilized for 5 minutes at 100°C before use.

1) Isolation of a streptomycin resistant strain

The parent strain, previously not exposed to streptomycin, was grown overnight in broth; one ml. of the culture was poured to agar plate containing 50 mcg. of streptomycin per ml. of agar. The plate was incubated for 48 hours at 37°C and an elliptic colony which appeared in the depth of the agar was inoculated into the broth containing 1000 mcg. of streptomycin per ml. After 48 hours the broth became a little turbid. A drop of the culture was again inoculated into the broth with the same streptomycin concentration. After 24 hours the bacteria had grown markedly. From the final broth culture a resistant strain to 1000 mcg. of streptomycin per ml. of broth was obtained. This strain also grows in the medium free of streptomycin and no change was noticeable in the resistancy to streptomycin even after several times repeated subcultures.

2) Isolation of a streptomycin dependent strain

The few colonies that appeared in the depth of agar plate containing 50 mcg. of streptomycin per ml., mentioned above, were incoulated radially around the cup, which was put in the centre of an agar plate and contained the streptomycin solution of 50 mcg. per ml. After the

incubation of the plate for 24 hours, the streptomycin dependent strain was sought among the growth in the vicinities of the cup by means of contrasting with the parent strain which grows in a far distance from the cup. The dependent strain thus obtained was used for the experiment after the subculture on an agar slant containing a relatively high concentration (500 mcg. per ml.) of streptomycin according to Pain and Finland.[7] The strain was found to be comparatively stable and the dependency was maintained also in a broth culture, i.e., the strain was inculated into the broths containing 500 mcg. of streptomycin down to 1.5 mcg. per ml. by two-fold dilution and into broth without streptomycin respectively. The shaking culture of these broths at 37°C for 24 hours proved that the growth gradually became better as the streptomycin concentrations increased from 7.8 mcg. to 500 mcg. The growth was exceedingly poor at 7.8 mcg. of streptomycin and below, while it was hardly noticeable in the broth containing no streptomycin.

3) Isolation of a chloramphenicol resistant strain

As in the case of the isolation of the streptomycin resistant strain, 24 hour culture of the broth was poured into an agar plate containing 45 mcg. of chloramphenicol per ml. of agar to obtain a resistant strain. After 48 hours no colony appeared. The chloramphenicol resistant strain differs from the streptomycin resistant strain in that it does not acquire a resistancy in one step as reported by Cavalli *et al.*[8] with regard to *E. coli*. Therefore, the resistancy in the broth was gradually enhanced from the minimum inhibiting chloramphenicol concentration of 6.3–10.5 mcg. per ml. for the parent strain until finally a strain with a resistancy of 470 mcg. of chloramphenicol was obtained. The resistant strain was preserved in the ordinary agar medium and furnished for experimental purpose with no change in resistancy.

Gocke and Finland[9] reported that a chloramphenicol dependent strain was isolated from a strain of the *Klebsiella pneumoniae*, which grew luxuriantly in a chloramphenicol concentration of 12–50 mcg. The author, however, could not obtain such a strain from both the sensitive strains of *E. coli* and *Klebsiella pneumoniae* preserved in author's laboratory, in spite of many efforts to get it.

Determination of Nucleic Acid Contents in each Strain at Different Culture Hours

Each strain obtained through the process mentioned above was cultured by shaking at 37°C. The broth used throughout the studies consists of 1 per cent meat extract, 0.5 per cent NaCl and 0.5 per cent peptone. 200 ml. of the broth was poured into each of the shaking flasks of 500 ml. capacity and sterilized. For the culture of the streptomycin

dependent strain streptomycin was added to a concentration of 10 mcg. per ml. All flasks were inoculated with one ml. of the bacterial suspension containing 1 mg. per ml. of saline solution for 6, 12 and 24 hour cutltures of each strain. The bacteria of 30 minute and 2 hour cultures were obtained from the culture based upon a large inoculum, that is, 10 ml. of the 24 hour culture broth was poured to 200 ml. of broth with exception of the dependent strain, the culture of which was obtained by inoculation in amount of 20 ml. The culture broth of each strain at 30 minutes, 2, 6, 12 and 24 hours were thus achived.

The further procedures were followed to the previous report[5];—formalin was added to each culture fluid at the rate of 0.5 per cent. After several hours, the bacteria collected by means of the de Laval centrifuge was washed with cold water. After the removal of acid-soluble and phospholipide fraction from the washed bacteria, the bacteria was dried and powdered.

A portion of the dried bacteria was used to determine the total nitrogen amounts by the micro Kjeldahl method and the remainder was dissolved in 1 *N* KOH solution. The bacterial solution was divided into RNA and DNA fraction. A liquot of the RNA fraction thus obtained was used to determine the amounts of the inorganic phosphorus according to Delory[10] and the remainder was used to determine of RNA amounts by two methods, viz., total phosphorus and pentose colour reaction. For the pentose colour reaction for RNA determination was used orcinol reaction,[11] using a commercial RNA preparation (Merck) as the standard by photoelectric colorimeter (Hitachi, Type TK-12OS). The results were multiplied by 10.6 to obtain the RNA value. Moreover, the RNA amount was obtained by substracting the inorganic phosphorus amount determined by the Delory method from the total phosphorus amount contained in the RNA fraction.

On the other hand, the total phosphorus amounts were determined in the DNA fraction and as in the case with RNA, the result was multiplied by 10.1 to obtain the DNA value in accordance with Schmidt. *et al.*[12]

The amount of the total nitrogen, RNA, DNA and inorganic phosphorus according to Delory, all of which was estimated quantitatively in the bacterial bodies of the parent, streptomycin resistant,—dependent and chloramphenicol resistant strains at various culture stages by above mentioned method is shown in Table I and Fig. 1 as the percentage of the dried weight of bacteria.

1) Total nitrogen amount: As can be noticed in the bacterial bodies of the parent, streptomycin and chloramphenicol resistant strains, the total nitrogen amounts increase markedly in the 2 hour culture, decrease in the 6 hour culture and remain almost constant in the 12 and 24 hour cultures. However, in the

TABLE I

Total Nitrogen and Nucleic Acids Amounts of each Strain during the Different Culture Hours (Expressed in percentage of bacterial dry weight)

	Culture hours	Dry weight of bacteria (g.)	N (%)	RNA (%)		Inorganic phosphorus (Delory %)	DNA* (%)	RNA/DNA
				phosphorus	pentose			
parent	24	1.13	12.01	9.86	10.44	0.022	3.29	2.99
	12	1.51	12.11	10.45	10.72	0.014	2.96	3.53
	6	1.29	13.63	8.65	10.21	0.037	2.64	3.27
	2	0.43	15.03	19.73	20.88	0.070	1.58	12.49
	0.5	0.31	12.19	19.22	19.50	0.033	1.71	11.23
Streptomycin-resistant	24	0.77	12.30	8.57	9.08	0.024	3.31	2.58
	12	0.94	12.84	10.43	10.95	0.018	3.02	3.45
	6	0.64	13.16	10.93	12.78	0.054	2.56	4.27
	2	0.63	14.03	18.36	19.86	0.037	1.55	11.84
	0.5	0.46	11.87	16.43	17.13	0.045	1.89	8.69
Streptomycin-dependent	24	0.58	11.35	10.91	10.77	0.014	3.10	3.51
	12	0.65	12.11	14.01	15.30	0.028	2.74	5.11
	6	0.14	12.76	14.69	15.21	0.050	1.48	9.92
	2	0.31	11.98	13.08	13.40	0.029	2.01	6.50
	0.5	0.23	11.02	12.12	12.33	0.017	2.54	4.77
Chloramphenicol-resistant	24	0.53	12.41	9.24	10.03	0.021	3.61	2.55
	12	0.48	12.79	10.43	10.73	0.020	3.45	3.02
	6	0.15	12.71	13.93	15.00	0.020	2.49	5.59
	2	0.29	14.65	18.50	19.54	0.037	2.04	9.04
	0.5	0.11	12.37	16.40	17.10	0.039	2.01	8.15

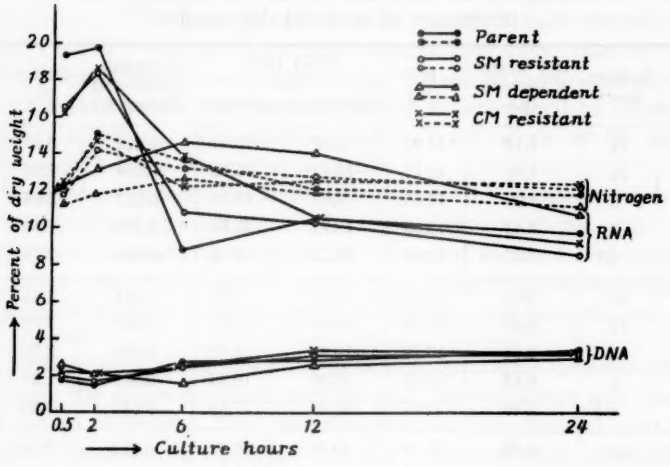
* Converted from phosphorus estimation.

case of the streptomycin-dependent strain total nitrogen amount does not increase sufficiently at the 2 hour stage and shows the tendency of a slight increase at the 6 and 12 hour stage, but the rate of the increase is less than that of parent and resistant strains.

2) RNA amount: The two methods applied in determining the RNA amount showed a slight difference for each strain at each culture stage, i.e., the value resulted by pentose reaction was slightly larger than that determined from the phosphorus amount. Particularly in the case where the increase of RNA was high, the difference also was distinct. However, the tendency of increase and decrease of these values was approximately the same.

In the parent, streptomycin-resistant and chloramphenicol-resistant strain the RNA amounts increase apparently in 30 minute and 2 hour culture stages and decrease thereafter. On the contrary, in the streptomycin dependent strain

Fig. 1. Graphic illustration of RNA, DNA and total nitrogen amounts in the bacterial cell of parent, Streptomycin-resistant, Streptomycin-dependent and chloramphenicol-resistant strains at the various culture stages.



it increases between 2 and 12 hours of incubation and decreases slowly until 24 hours. The increase and decrease were, however, not so rapid as observed in the parent and resistant strains.

3) The inorganic amount estimated by Delory's method; the amounts detected from the RNA fraction are very minute for each strain at various culture stages. However, the inorganic phosphorus amount appears also to increase when RNA contents in the bacteria increase.

4) DNA amount; the total phosphorus amount of DNA fraction was converted to DNA value as in the case of RNA. Hardly no difference was noticeable in the values among the parent, streptomycin resistant, dependent and chloramphenicol resistant strains.

Internal Free Amino Acids Detected by Paper-chromatography

From the results of determination of nucleic acid and total nitrogen amounts, a ditinctly few and delayed increase of RNA and nitrogen in the streptomycin dependent strain was ascertained in comparison with those of other strains.

Clapper *et al.*[13] stated that a considerable amount of free glutamic acid was found in all of the trained sulfathiazole, aureomycin and penicillin resistant strains of viridans streptococci tested, but not in the parent strains. Therefore, anticipating that some difference in the quality and

quantity of free amino acids within the bacterial bodies of streptomycin-resistant and -dependent and partent strains may exist, the author attempted to detect the free amino acids from these strains by paper chromatographic method.

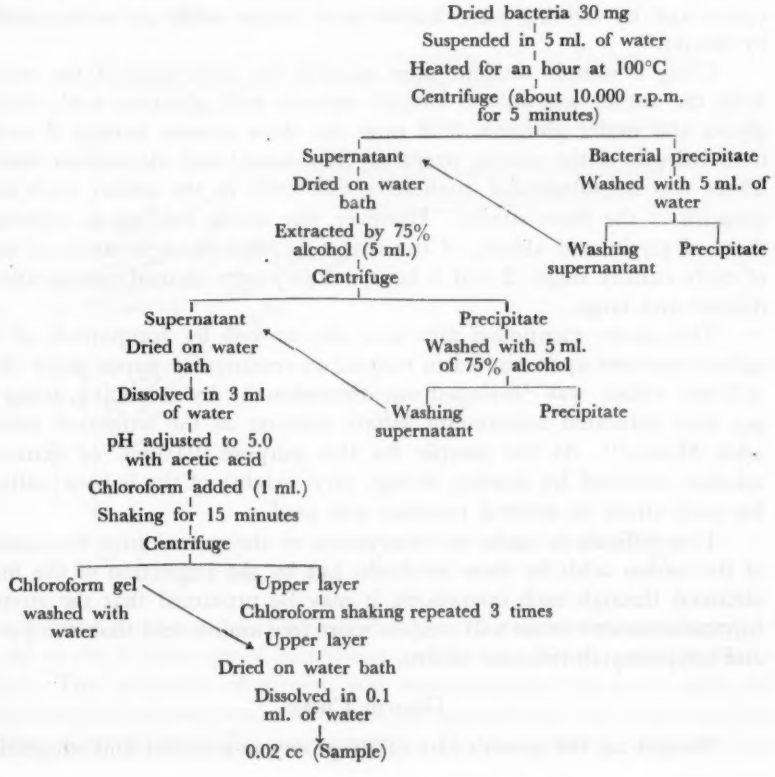
Samples: 2 and 6 hour culture of the parent, streptomycin-resistant and -dependent strain obtained by shaking culture for each at 37°C were washed with saline and water until the broth constituents disappeared (negative on ninhydrin reaction) and dried.

Further procedures according to Awapara[14] is shown in Table II

Thirty mg. of dried bacteria were suspended in water and extracted by heating it in neutral reaction. After the extracted solution was dried

TABLE II

The Procedures for obtaining the Sample for Paperchromatographic Analysis



on water bath, the dried mass was dissolved in 75 per cent ethanol solution. The ethanol soluble part was dried on the water bath. Next, the dried mass was dissolved in water and after the protein was removed by chloroform shaking-method the solution was again dried. The dried mass was dissolved in 0.1 ml. of water, 0.02 ml. of which was used as a sample for paper chromatographic analysis.

Irrigation and development of paper chromatograph: Two dimensional developing by ascending method was adapted in accordance with the method of Kimura and Morikawa.[15] For the primary and secondary dimensional irrigation, 75 per cent phenol solution and the supernatant of butanol, water and acetic acid mixture (4:5:1) were used as the irrigation solvents respectively. 0.25 per cent ninhydrin butanol solution was used as the developing reagent.

Identification: Each amino acid spot on the paper chromatographic map was developed by ninhydrin reaction and identified by the pure amino acid map, which was run under the same condition, by the Rf values and by crossing some known pure amino acids as recommended by Satake.[16]

Using a sample amount most suitable for separation of the amino acids the author was able to identify aspartic acid, glutamic acid, alanin, glycin and under glutamic acid from the three strains, namely 2 and 6 hour cultures of the parent, streptomycin-resistant and -dependent strains. There was no substantial qualitative difference in the amino acids constituents of the three strains. However, the amino acid spots, especially those of glycin and alanin, of the streptomycin-dependent strain at each of early culture stage (2 and 6 hour culture) were stained comparatively distinct and large.

The above mentioned fact was also proved by comparison of the colour reactions upon ninhydrin reagent on rectangular paper sheet (25.5 $\times$ 3 cm) which was developed one-dimensionally by ascending, using 60 per cent saturated ammonium sulfate solution as the irrigation solvent after Miura.[17] As the sample for this purpose, 0.03 ml. of extracted solution obtained by heating 50 mg. (dry weight) of the 6 hour cultures for each strain in neutral reaction was used.

It is difficult to make an observation on the quantitative relationship of the amino acids by these methods, but by the inspection of the maps obtained through such operations, it may be presumed that the streptomycin-dependent strain will contain more free amino acid than the parent and streptomycin-resistant strains.

Growth Curve

Studies on the growth rate of streptomycin-resistant and -dependent

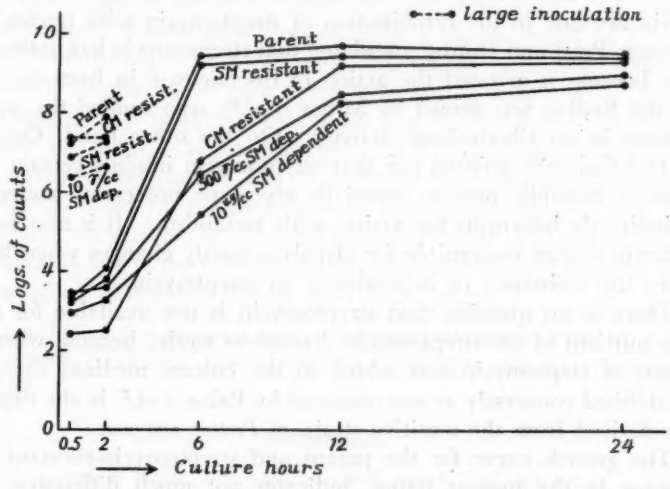
strains derived from sensitive strains of *E. coli* and *Proteus morgani* has been reported by Paine *et al.*[7] and Smith *et al.*[18]

The author also compared the growth rates of the streptomycin-resistant and -dependent, chloramphenicol-resistant and parent strains in shaking cultures.

The inocula for 200 ml. of broth culture of each strain was the same as in the foregoing experiment of this paper. One ml. of each of the 24, 12, 6, 2 hour and 30 minute culture broth was diluted serially to a suitable cell population in saline solution (10–fold dilution) and one ml. of each of the diluents was poured into 5 agar plates respectively. After the plates were incubated for 24 hours, the number of the growing cells were counted. In the case of the streptomycin-dependent strain both, the broths containing 10 and 500 mcg. of streptomycin per ml. were used respectively and the count was done on a respective pour agar plate containing same amount of streptomycin per ml. of agar.

The viable cell number per ml. of the culture broth of each strain during different culture hours is expressed in logarithm in Fig. 2.

Fig. 2. Growth rate of each strain by shaking at 37°C. The logarithms of the numbers of colonies are plotted against time.



For the parent strain, it is plain that the cell numbers increase markedly up to the 6 hour age of incubation and become nearly constant thereafter. This tendency of growth was approximately the same with the streptomycin resistant strain although the growth rate was somewhat less being compared with the parent strain. On the contrary, streptomycin-

dependent strain cultivated in 10 mcg. concentration of streptomycin some growth was noticeable during 2 to 12 hour culture stage and slight increase was conceivable at the 24 hour culture being compared with the 12 hour culture. The increase in cell number of streptomycin dependent strain cultivated in 500 mcg. concentration of streptomycin exceeded that cultivated in 10 mcg. concentration of streptomycin, but the growth rate was less than that of the parent and streptomycin resistant strain. At 30 minute and 2 hour culture stage the increase of the cell number of chloramphenicol resistant strain was scarcely recognized and though there was some growth in the period between the 2nd and 12th hour of incubation, it was less than that of the parent and streptomycin resistant strain.

The tendency of the growth in the case of large incoulation at 30 minute and 2 hour culture stages was nearly the same as that of small inoculation, although it was somewhat higher elevated in total in the former than in the latter case.

Discussion

Regarding the mechanisms of antimicrobial action of streptomycin Cohen[19] and Donovick *et al.*[20] stated that actions of streptomycin against bacteria are due to the combination of streptomycin with nucleic acids. Moreover, Pratt and Dufroy postulated that streptomycin has antibacterial action because it arrested the action of ribonuclease in bacteria. However, this finding was denied by Miura *et al.*[21] who studied the action of streptomycin on ribonuclease activity. On the other hand, Oginsky *et al.*[22] and Geiger[23] pointed out that streptomycin inhibits certain enzym systems or metablic process, especially glycolytic process of bacteria and thus indirectly interrupts the amino acids metablism. It is also said that the enzym system responsible for glycolysis partly changes when bacteria acquire the resistancy or dependency on streptmycin.

There is no question that streptomycin is not available for growth as the nutrient of the streptomycin dependent strain, because when large amounts of steptomycin was added to the culture medium the growth was inhibited conversely as was observed by Paine *et al.*[7] in the dependent strain derived from the sensitive strain of *Proteus morgani*.

The growth curve for the parent and streptomycin-resistant strain, as shown in the present paper, indicates not much difference, except that the growth of the latter is slightly retarded. With regards to the total nitrogen, RNA and DNA contents, there was also hardly distinct difference. On the contrary, the growth of the streptomycin dependent strain was slack, the total nitrogen contents less than that of the parent and streptomycin-resistant strain, the increase of the RNA contents was

not marked too. Also the bacterial bodies of streptomycin dependent strain obtained in the early stage of growth seemed to contain more free amino acids than that of the parent and streptomycin-resistant strain.

By using *Bacillus cereus* in their experiment, Henry *et al.*[24] recognized that total nitrogen and phosphorus amounts of the bacteria decrease when the growth was arrested by streptomycin.

Streptomycin is required for the growth of streptomycin-dependent strain and in suboptimal concentration of streptomycin unsuitable for growth, its growth is checked. As a result it may be presumed that the synthesis of RNA and subsequently the synthesis of protein is not carried out sufficiently.

As for the chloramphenicol-resistant strain, the resistancy is acquired gradually. The results of experiment with chloramphenicol resistant strain showed that the extent of growth was less than the parent strain, but as for the total nitrogen, RNA and DNA content of the bacteria at various culture stages, there was no difference when compared with the parent strain.

Summary

1. Streptomycin-resistant, chloramphenicol-resistant and streptomycin-dependent strains were isolated from a parent strain of *E. coli*.

2. Total nitrogen, nucleic acid, free amino acid and viable cell number were determined in each strain at various culture stages. Streptomycin-resistant and chloramphenicol-resistant strains showed a tendency of increase and decrease in total nitrogen and RNA amounts approximately similar as the parent strain. On the contrary in a streptomyin dependent strain which shows a retarded growth, the total nitrogen and RNA amounts did not increase to the same rate and extent as above and it may be presumably due to the fact that the protein synthesis of the strains would not proceed so sufficiently during the culture stages.

In concluding this paper, I wish to express gratitude to Prof. M. Kuroya for his generous guidance throughout the experiment and for reviewing the manuscript.

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On an Undescribed Gingival Symptom observed in Allergic Skin Disease Patients

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Introduction

Some years ago, we[1] came to notice a hitherto undescribed symptom on the gingivae of some patients suffering from erythema induratum Bazin. At first, we studied such symptoms under the impression that they might be related with some tuberculous disorder, but the research proved in vain. Upon collecting more clinical data, we found that symptoms were apt to appear frequently in cases of some certain types of allergic skin diseases. Accordingly, we experimented with rabbits with induced anaphylaxis and succeeded in causing the appearance of analogous symptoms.[2] We will report on our observations and experiments in the following.

Clinical

Observed cases

On clinical findings of 6 cases, we may present here 2 examples in detail and the others will be showed as Table I.

Case 3. Name: Y. S.; female, aged 21; first examination: May 21, 1952; diagnosis: erythema induratum Bazin. Findings of gingiva: Stipplings on the attached gingiva rather limited in number. Wiped dry with cotton swabs, flat and rather rough surfaced plaques with slightly depressed circumference were found, 3 in the maxilla and 1 in the mandible, at the edge of the attached gingiva almost touching the free gingival groove, on the interdental papillae $\underline{321}|$, $|\underline{12}$ and $\overline{21}|$, that between $\underline{32}|$ being about half grain-size and the largest, the others being about 1×8 mm. Observation with a slit lamp microscope showed that they also contained numerous pinholes stipplings. On July 14, when the disease

on her legs was cured, the plaque between 21| became blurred and the others vanished, except that between 21| which persisted in the same appearance.

Case 6. Name T. M.; male, aged 26; first examination: July 28, 1953; diagnosis: toxicodermia due to fried oysters. Findings of gingiva: Stipplings disseminated on the attached gingiva, especially crowded on the papilla between 21| (Orban's localized stipplings). Observation after wiping dry with cotton swabs revealed existence of a slightly rough and milky white surfaced plaque, 2 mm. in veritcal and 1 mm. in horizontal dimensions, slightly depressed at the circumference and raised at the center, looking like an inlaid piece of an ellipsoid surface, on the attached gingiva of the papilla between |12, close to the free gingival groove and somewhat nearer to the tooth |2. Very minute stipplings were observed densely spotting the plaque, barely visible with the naked eye (Fig. 1).

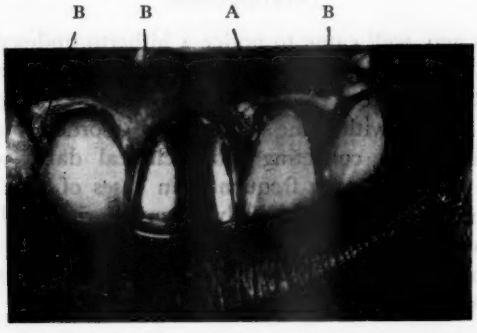

Fig. 1. A: Typical plaque; photographic contrast emphasised by rubbing in Indian ink. B: Localized and scattered fine stipplings (Orban).

Of the findings described above, we will hereinunder call the circular or elliptic plaques with pinhole stipplings barely visible with the naked eye the "typical plaque," in contradistinction to the commonly observable Orban's localized stipplings and etc.

Histological observations

1) Typical plaques

Case 7. Name: K. S.; female, aged 21; diagnosis: erythema induratum Bazin and phlebitis nodosa tuberculosa. Findings of gingiva: On March 23, 1952, observed typical plaques on the papillae between teeth 21|1 and |12, on April 17 between 321| and |23 and on April 21 between |12. We excised the old typical plaques between 321| and |23 on April 17 and the fresh plaque between |12 on April 21. They were

TABLE I

Clinical Findings

Case no.	Sex	Age	Diagnosis	Findings of the "typical plaque"		Another findings
				Localities	Shape and Size	
1	f	31	erythema induratum Bazin	On the attached ginviva of the 21| interdental papilla adjacent to the free gingival groove	Slightly concave plaque of half grain size, whitish and rough surface with barely visible pin holes	Orban's fine stipplings scattered all over the surface of gingiva
2	f	21	erythema induratum Bazin	At the base of the interdental papilla of |34	A smooth, milky white plaque of ca. 1.5 cm. in diameter, giving the appearance of an inlaid spherical surface with barely visible pin holes	Orban's stippling disseminated all over the surface of the attached gingiva of maxilla. Interdental papillae showed generally the blood statis. Dental calculi somewhat overgrown
4	f	30	erythema annulare centrifugum Darier	At the |12 interdental papilla about 2 mm. far from the free gingival margin	A sharply outlined disc of ca. 1.5 mm. in diameter. The other findings were the same as mentioned above.	
5	f	26	pulmonary tuberculosis	At the base of the papillae between |12 and |23	Nearly circular plaque, 1 mm. in diameter. The other findings quite corresponded to the above description.	Orban's stipplings disseminated all over the attached gingiva and the localized lies on the interdental papillae of 1|1.

fixed in formalin, imbedded in paraffin, stained with hematoxylin-eosin and van Gieson method and subjected to microscopic examination. The microscopic preparations cited hereunder were prepared by the same process throughout. The observations obtained in this case are outlined as follows.

a) Fresh typical plaque: The epithelium was seen to proliferate rather irregularly in the deeper layers, and showed elongated or drop-formed hollow spaces here and there on the surface, generally coinciding with the rete cones, but the cells composing the wall of hollow spaces were not different from the surface layer of the epithelium. Vascular dilatation was conspicuous in the superficial layer of the lamina propria, with cell infiltration of from slight to extremely high grade but limited to the perivascular regions, consisting of round cells in the main, with an admixture of plasma cells and polynuclear leucocytes; it becomes gradually suppurant as the tip of the papilla is approached.

b) Old foci: The epithelium proliferated inwards, protruding long and either broad or narrow in width. Depressions on the surface of the epithelium fall together with vast epithelial pegs in general, the larger of the depressions containing epithelial pearls. Dilatation of blood vessels is frequently found in the lamina propria, and some plasma cells and small round cells are found perivascularly or in diffusion.

As for the other eight examples, including 4 cases of the typical plaques, 2 cases of Orban's localized stipplings and 2 cases of the coexistence of the typical plaque and Orban's localized stipplings, we tabulated the essentials of their histological findings en bloc (Tables II & III).

Summary of symptoms

1) Clinical findings: Observed upon wiping the surface of the gingivae dry with cotton swabs, plaques generally milky white, but sometimes in the color of the healthy gingiva, are found formed in most cases on the attached gingiva, close to the free gingival groove, but sometimes going over to the free gingiva of the interdental papillae, 1–1.5 mm. in vertical and 0.8–1 mm. in horizontal length, circular or elliptical in shape, with the circumferences depressed slightly and somewhat raised at the center in most cases, giving the appearance of inlaid pieces of spherical or ellipsoid surfaces, but sometimes rough-surfaced, wherein numerous minute pinholes barely visible with the naked eye are observed. Such observations may be clearly verified with a slit lamp microscope, and the pinholes are seen as red-colored (Fig. 1). The number of plaques per capita was from 1 to 7, but in most cases they counted only 1 to 2, averaging 1.83 in the observed cases (Table IV).

2) Locality: As shown in Table V, a decided majority was found on the papillae between the incisors and cuspids of the maxilla, followed

TABLE II

Histopathological Findings of the "Typical Plaque" and Orban's Localized Stipplings

Case no.	Sex	Age	Diagnosis	Surface of epithelium	Epithelial layer	Lamina propria
1) Typical plaque						
8	f	21	pulmonary tuberculosis	depressions corresponded to vast epithelial cones	proliferated to the depth in long pegs, either broad and narrow	vascular dilatation and capillarization, diffuse and localized infiltration consisting of plasma cell and lymphocyte
9	m	22	pulmonary tuberculosis	gentle depressions over vast rete cones	irregular elongated rete cones	slight, perivascular infiltration of round cell with light dilatation of blood vessels
10	m	35	pulmonary tuberculosis	similar to the above	similar to the above	diffuse and occasionally small foci formed infiltration of plasma cell and lymphocyte
11	m	20	pulmonary tuberculosis	similar to the above	similar to the above	edema adjacent to apecis of rete cones, circumscribed lymphocytic infiltration
2) Orban's localized stipplings						
12	f	22	in normal health	many elongated depressions with occasionally exposed lamina propria	rete cones almost gone	dilatation of blood vessels, foci of lymphatic infiltration
13	f	25	in normal health	larger but shallow depressions	no change	slight infiltration of round cells

TABLE

Histopathological Findings in the Case of Coexistence of

Case no.	Sex	Age	Diagnosis	Typical plaque		
				Surface	Epithelium	Lamina prop.
14	m	25	epididymitis tuberculosa	drop-shaped depressions corresponded to vast rete cones	proliferated in deeper layer	diffuse and localized infiltration of plasma cell and lymphocyte
15	f	25	pulmonary tuberculosis	depressions corresponded to vast rete cones	a little irregularly proliferated in deeper layer	infiltration of plasma cell only

by the papillae between the incisors of the maxilla on both sides; in all cases the typical plaque was seen on the interdental papillae from incisor to premolar both of the maxilla and the mandible. Rarely the location was outside the papillae. Plaques in the maxilla were more frequent than in the mandible.

3) Duration: As in most cases the plaques had been formed before the clinical examination, the precise duration of their existence could not be measured, but we observed that they disappeared in around one week by the two dietary toxicodermia cases, and in most cases plaques disappeared with the cure of exanthemata, excepting the cases of erythema induratum Bazin where the plaques were found to persist after the effacement of eruptions and unable to be followed up to the last.

4) Age and sex: There seems to be no difference in incidence by sex. In age, youth and early maturity seemed to predominate, but this observation is not based on the total number of patients classified by the types of diseases, so we cannot claim finality for it.

5) Frequency by various disease: Among the tuberculous disorders, erythema induratum Bazin patients show a very high frequency, as shown in Table VI. Among the oridinary allergic skin diseases, erythema nodosum, erythema annulare centrifugum Darier and pityriasis rosea Gibert claim the highest incidence, followed by toxicodermia and erythema exsudativum multiforme (Table VII). No such plaques could be observed in other skin diseases and veneral and urologic disturbances

6) Distinction from other similar findings: Scattered stipplings are normally seen on the attached gingiva, and they are often seen grouped in a limited area there, mostly near the free gingiva, especially frequently when artificial metal crowns have been fitted. These grouped stipplings were called "localized stipplings" by Orban and can be distinguished from the typical plaques by the following criteria: 1) The localized stipplings may be identified without trouble but the typical plaques cannot

III

the "Typical Plaque" and Orban's Localized Stipplings

Orban's localized stipplings		
Surface	Epithelium	Lamina propria
depressions slight	rete cones proliferated rather irregularly	slight infiltration of lymphocyte
depressions slight	rete cones ill developed	localized infiltration of plamsa cell with a few of lymphocytes

be observed before freeing the gingiva from mucus and saliva with a cotton swab and dry it. 2) The typical plaques are either round or elliptical, while the localized stipplings are irregular in shape. 3) The pinholes on the typical plaques are so minute that they can be barely seen with the naked eye, while the localized stipplings may be seen without difficulty. 4) Most of the typical plaques are somewhat humid milky white in color and show the appearance of being rough-surfaced or granulated, but never the localized stipplings. 5) The typical plaques are circumscribed by slightly depressed, clear-cut edges, and have somewhat raised centers, so that they give the appearance of inlaid pieces of spherical or ellipsoid surfaces, but localized stipplings are different. 6) The typical plaques are temporary in any case, though their duration may differ with the type of the skin diseases, but the localized stipplings are persistent. 7) Histologically, there are the following differences: i) On the surface of the mucosa, the depression begins in a drop-like form with a smooth boundary, growing deeper and sometimes forming a pearl in it, by a typical plaque, while by a localized stippling, the depression is irregular in form and shallow in most cases. ii) By a typical plaque, the epithelium conspicuously proliferates in the deeper layers, the rete cone grows long and sometimes thick and sometimes thin, and the papillae of the lamina propria are found protruding in long, thin and irregular threads samong the pegs. By the localized stipplings, however, the epithelial proliferation in the deeper part is slight or the pegs are lost altogether. iii) Cell infiltration of the lamina propria is varied in its intensity by both the typical plaque and the localized stippling, from case to case, but by the former, it consists generally of plasma cells and lymphocytes, with an admixture of polynuclear neutrophiles in the early stage, while by the latter, plasma cells are rarely found in it. However, in Case 16 above, where both typical plaque and localized stippling were found, infiltration by plasma cells was found by both, so that the distinction by infiltration cells cannot be

TABLE IV

Distribution by Skin Diseases

Case No.	Sex	Age	Diagnosis	Localities
1	m	37	toxicodermia	1 2, 1 2
2	m	24	„	2 1
3	m	29	„	2 3
4	m	60	„	1, 1 1, 1
5	m	57	„	3 2
6	f	22	„	2, 1 1 2, 2 1 1
7	m	25	pityriasis rosea Gibert	3 2
8	m	25	„	2 3
9	m	29	„	1 2 3
10	m	31	erythema nodosum	3 2 1 1 2 3, 1 2, 1 2
11	m	25	„	2 1
12	f	20	erythema exsudativum multiforme	2 1
13	f	23	„	4 3, 3 4, 3 2, 3 4
14	f	22	erythema annulare centrifugum Darier	1 1
15	f	18	erythema induratum Bazin	2 1, 3 2
16	f	21	„	3 2, 1 2, 3 2
17	f	28	„	2 3, 3 2, 2 3
18	f	21	„	3 2 1, 1 2
19	f	25	„	1 1 2 3
20	f	18	„	2 1
21	f	29	lupus vulgaris	2 1, 1 2
22	m	18	pulmonary tuberculosis	2 1, 1 2
23	m	20	„	2 3
24	m	35	„	2 3
25	f	21	„	6
26	f	22	„	3 2
27	m	24	„	3 2
28	m	24	„	3 2
29	m	29	„	3 2
30	m	32	„	2 3
31	f	25	„	1 2
32	f	26	„	1 2 3
33	m	30	„	1 1
34	m	25	dermatitis acuta	2 3
35	m	16	syphilis congenita	2 3

TABLE V

Distribution Frequency

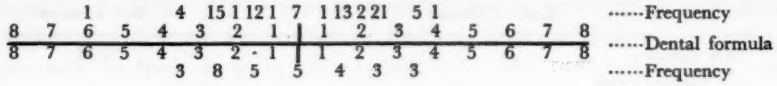

TABLE VI

Frequency in Tuberculous Diseases

Kind of diseases	Nos. of cases
Pulmonary tuberculosis	12/169
Tuberculosis of children	1/2
Pleuritis	0/2
Tuberculosis of kidney	1/4
Epididymitis tuberculosa	0/1
Erythema induratum Bazin	16/21
Lupus milliaris disseminatus faciei	0/1
Acne rosacea like tuberculid	0/1
Lupus vulgaris	1/1
Total	31/195

TABLE VII

Frequency in Allergic Skin Diseases

Kind of diseases	Nos. of cases
Eczema acutum	1/18
Eczema chronicum	0/2
Toxicodermia	9/18
Urticaria	2/7
Lichen urticatus	0/5
Strophulus infantum	0/1
Erythema exsudativum multiforme	2/4
Erythema annulare centrifugum Darier	2/2
Pityriasis rosea Gibert	4/5
Erythema nodosum	2/2
Erythematodes discoides	1/8
Total	23/72

deemed as of importance. Moreover, since it has been reported[3] that infiltration by inflammation cells can be proven microscopically in apprently healthy gingivae, the only distinction that can be asserted as final in histological tests boils down to the marked proliferation of the epithelium.

TABLE VIII

Frequency in Another Diseases

Kind of disease		Nos. of cases
Skin diseases		0/43
Lichen Vidal	3	
Dermatitis seborrhoides	7	
Ariboflavinosis	1	
Acne vulgaris	?	
Acne rosaccea	1	
Purpura annularis teleangiectodes	1	
Schamberg's disease	1	
Porokeratosis	1	
Atheroma	1	
Osmidrosis axillae	2	
Riehl's melanosis	1	
Chloasma	1	
Leucoderma pseudosyphiliticum	1	
Gingivitis erosiva	1	
Combustio bullosa	1	
Herpes zoster	2	
Condyloma acuminatum	1	
Furunculosis	5	
Trichophytia pompholyciformis	6	
Trichophytia eczematosa	1	
Scabies	2	
Venereal diseases		1/24
Genitourinary diseases (non tuberculous)		0/8
Other disease		0/2
Without disease		0/30
Total		1/107

ANAIMAL EXPERIMENTS

Seeing that the above gingival symptoms were frequently found among patients with allergic skin diseases, we made animal experiments for further study, using rabbits in which anaphylaxis had been induced.

Materials

Rabbits of about 3 kg. body weight fed with a prescribed diet for more than one month were used. For sensitization to and reinjection of serum anaphylaxis, horse serum diluted down to twice its volume with disinfected distilled water was employed. Neoehramizol (Neoarsenobenzol V.P.J.), in doses of 0.15 g. dissolved in 7.5 cc. of disinfected distilled water,

mixed with an equal volume of horse serum was used as antigen against anaphylaxis, and 0.15 g. of neoehramizol dissolved in 10.0 cc. of disinfected distilled water for reinjection. These materials were always stored in ice box. Horse serum, donated by the Institute of Infectious Diseases through the kind office of Prof. S. Kudo of Tokyo University, was used in fresh state in all experiments. We herewith express our hearty thanks to Dr. Kudo.

Method

0.2 cc. of the twice diluted horse serum to four rabbits and 0.2 cc. of neoehramizol solution with horse serum added to three others were given as intracutaneous injections in the gluteal region for the purpose of sensitization. Two weeks thereafter, for the process of reinjection 2 cc. of diluted horse serum was injected carefully into the auricular vein of the former and 2 or 4 cc. of neoehramizol solution (i.e. 0.03 g. or 0.06 g. of neoehramizol) to the later.

Results

About 4–20 hours after the reinjection all the seven rabbits subjected to the experiments showed slight unevenness on both lateral sides of the incisor which developed into many very conspicuous small depressions in 3 days or thereabout. Then they gradually lost distinctness and faded out within 2–3 weeks, except in one case, where a depressions persisted for 25 days. Upon reinjection, the position for sensitizing injection showed erythema and the auricles cyanosis. One of the animals died on the next day of the reinjection (Table IX).

TABLE IX

Experiment with Rabbits

No. of rabbit	Sensitization	Reinjection	Findings at the sensitized locus	Changes of gingiva	NB.
1	horse serum	horse serum	reddening	+	
2	"	"	"	+	
3	"	"	"	+	
4	"	"	"	+	died on the next day of the reinjection
5	horse serum and neoarsenobenzol	neoarsenobenzol 0.03 g	"	+	
6	"	"	"	+	gingival changes disappeared after 26 days
7	"	" 0.06 g	"	+	

Histological observations

The affected sites of the gingivae of the rabbits were excised at fixed intervals and after formalin fixation and paraffin imbedding, were stained with hematoxylin-eosin and van Gieson's method for microscopic examination.

3 hours after reinjection: Edema and dilatation of blood vessels conspicuous; perivascular infiltration of round cells in rare case. Basal cells of the local epithelium formed several layers which gradually passed over into prickle cell layer. These cells became more strongly stainable and the outer prickle cells contained vacuoles.

24 hours after: Beside the above described changes, the epithelium was observed proliferated in the deeper part, the epithelial pegs arranged irregularly, and amitosis of the epithelial cells in the deeper layers is noted strikingly though not so rich in number, together with vacuolation. The surface of the mucosa showed depressions roughly corresponding to the major changes of the epithelium.

2 days after: Edema and dilatation of blood vessels were yet observable in a high degree. A very slight perivascular infiltration of round cells was also seen in lamina propria. The part of the superficial layer of the epithelium responding lightly to eosin has become to form more and more thickened and the deeper layers showed figures of vacuolation and amitosis.

3 days after: Change of lamina propria not so much different from the above; the superficial eosin-stainable layer of the epithelium grown thicker yet and in part divided only by a few cell layers from the papillae of the lamina propria, where the formation of depressions on the surface of the epithelium was distinct; proliferation of epithelium in the deeper layers intensfied; epithelial pegs grown vaster; amitosis apparent; the papillae of lamina propria become elongated and irregular.

4 days after: The surface of the epithelium rich in unevenness; epithelial proliferation in deeper layers conspicuous and irregular, accompanied by mitosis and amitosis of cells; lamina propria sometimes intruding deep into epithelium; depressions on the surface of epithelium found over such spots in general; the lightly eosin-affine layer of the epithelium reduced in thickness; between this layer and the basal cell layer a noneosin staining prickle cell layer grew gradually; edema and vascular dilatation in the lamina propria decreasing and only a slight round cell infiltration observable in the superficial layer of the lamina propria perivascularly.

A week after: Surface of epithelium depressed here and there; epithelium partially proliferated in deeper layers and epithelial pegs

irregular. Edema remaining in the superficial layer of the lamina propria; blood vessels dilated; slight proliferation of endothelial cells of blood vessels and few perivascular round cell infiltration observed.

Later on, the proliferation of epithelium vanished, the epithelial pegs recovered regular arrangement, the surface depressions faded, the edema in the papillary layer diminished gradually, and a follow-up for four weeks showed that the vascular dilatation and the perivascular infiltration of round cells progressively lost in strength, so that in four weeks, the only signs left were proliferation of vascular endothelium cells and obliteration of blood vessels in the lamina propria.

Summary and Discussion

In examining the gingivae dried with cotton swabs of skin disease patients, such as erythema induratum Bazin, erythema nodosum, erythema annulare centrifugum Darier, pityriasis rosea Gibert, toxicodermia, erythema exsudativum multiforme and so on, we found some opaque or normal colored, somewhat rough-surfaced plaques, on the papillae of the attached gingiva chiefly between the incisors and cuspids, sometimes running over into and sometimes adjacent to the free gingival groove, from millet-grain to half rice-grain size, with slightly depressed and clear-cut circumferences and raised centers, looking like inlaid pieces of spherical or ellipsoid surfaces, but in some cases with flat surfaces, wherein numerous tiny stipplings barely visible with the naked eyes are seen which are revealed as red colored pinholes by looking through a slit lamp microscope. To our knowledge, such signs have never described to date. Based upon the fact that such plaques have been most frequently observed among allergic skin disease patients, we made the experiment of inducing horse serum or salvarsan anaphylaxis in rabbits and examining their gingivae, when it was found that these rabbits showed no formation of such typical plaques as described above, but there appeared some unevenness on the gingivae of the lateral side of their incisors within 3 to 20 hours after the treatment, which developed into small stipplings in 2 days or so. This result seems to justify us in deeming such plaques as a gingival manifestation of allergic diseases. We must point out that our investigation on the frequency by various types of allergic diseases cannot claim an exhaustiveness, and we are not yet in the position to say anything on the cause or the mechanism of formation of this symptom, but we may tentatively propose the supposition that the plaques are formed accompanying comparatively acute incidence of allergy. This fact can be deduced from the fate of the plaques by patients of such diseases beginning and ending in consonance with the progress of the main complaints and of that of the analogous symptoms appearing in experimentally anaphyl-

axized rabbits.

Histopathologically speaking, intense proliferation of epithelium in its deeper layers was found characteristic to both the clinically observed and the experimental cases. This anomaly seems to stand in a relation with the formation of spherically surfaced plaques and of small stipplings thereon. Indeed, the maximum of the cell proliferation in the experimented rabbits were found to coincide with the completion of stipplings on the third day after the treatment. In fact Nakayama *et al.*[4] have interpreted the formation of epithelial pearls in the oral mucosa of human embryos and new-borns as due to the atrophy of previously proliferated epithelial cells or to the pressure occasioned by the proliferation. And Orban[3] observed, in connection with the mechanism of stippling formation, that " these depressions are brought about by connective tissue fibres attaching the gum to the underlying alveolar bone as if each depression were anchored down by fibre bundle," citing King's remarks and based on his histological examination of the direction of connective tissue fibres. We found only one case of pearl formation in our clinical examination, but from the facts that proliferation of the epithelium was observable in all cases clinically and experimentally, and that in the experiments with rabbits edema was always frankly evident, we presume that the formation of the " typical plaque " may be most plausibly explained by the pressure exerted by the epithelial proliferation and edema plus by the traction of connective tissue fibres.

In the above, we have followed Orban in the use of terminology concerning the topographical anatomy of the gingiva.

We are indebted for the observations of gingivae to the kind suggestions of Prof. R. Higaki and Lecturer J. Ishikawa of Tokyo Medico-Dentistry University, and to the instructions of Asst. Prof. G. Ishikawa of the same University for the histopathological findings. Mr. F. Hirose, Expert Photographer of the same assisted us in the preparation of the photographic illustration of affected gingivae. We herewith express our hearty thanks to these gentlemen.

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